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SCHOOL OF PUBLIC HEALTH

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ENVIRONMENTAL MICROBIOLOGY

AS RELATED TO PLANETARY QUARANTINE

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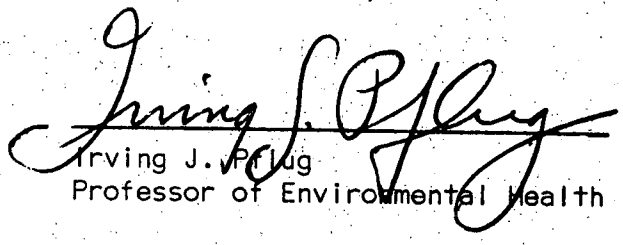

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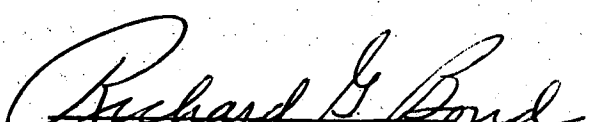

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INTRODUCTION

This report covers research activities during the period June 1, 1971, through November 30, 1971, for the project entitled "Environmental Microbiology as Related to Planetary Quarantine." These studies were conducted by the Division of Environmental Health, School of Public Health, at the University of Minnesota under the auspices of the National Aeronautics and Space Administration and Lawrence B. Hall, Planetary Quarantine Officer.

This is the seventh semiannual report of progress on NASA project NGL 24-005-160. A limited number of earlier reports on this project are available for those who do not have a complete set but who wish to obtain all of our procedures and results in a given area.

The results of our studies to determine the effect of soil particle size on the survival time at 125°C of the microflora associated with these particles are discussed in this report. The data suggest that longer survival times exist for the microflora associated with the larger particles. It seems appropriate to briefly review dry heat sterilization and then fit these data into that picture.

One basic characteristic of sterilization processes is that the intensity of the process necessary to produce a given probability of sterility is a function of the microbial load and the physical and chemical characteristics of the unit being sterilized. Dry heat sterilization processes appear to fit the pattern; for organisms in garden soil or Cape Kennedy soil, very long sterilization cycles at high temperatures are required to produce the same probability of sterility that can be achieved for naked organisms with a short sterilization cycle. Therefore to sterilize space hardware, there is a wide range of sterilization cycles possible depending on the nature of the microbial contamination. However, in all cases the sterilization cycle is determined by the nature of contamination. In designing a heat sterilization cycle for space hardware, the quantity of contamination on the hardware can be measured and then the cycle designed or the sterilization cycle (which will place limits on the contamination) can be specified after which the manufacture and assembly conditions must be designed to meet the contamination requirements.

The studies that have been carried out indicate that microorganisms associated with soil are difficult to kill and that organisms associated with large particles are harder to kill than those associated with small particles. It is our opinion that all of the NASA dry heat research points out that sterilization requirements increase as the level of contamination increases. Soil particles and their accompanying microflora are the most critical contaminants.

Research supported by the NASA Planetary Quarantine program has made a major contribution to the overall knowledge of the dry heat destruction of microorganisms. However, these data are also valuable to other scientific disciplines. At the present time researchers that are working with biological materials, i.e., food and fiber systems that are subjected to heat treatment at low water activities are examining the data that has been generated by the NASA Planetary Quarantine research program to learn more about how moisture may affect biological reactions.

There are still a number of problems relating to the dry heat resistance of microorganisms that need to be evaluated; one of these relates to the systems being used for gathering dry heat resistance data. Researchers in the several laboratories participating in the NASA program for developing basic data for the design of dry heat sterilization processes have all been working with the same species of microorganism, Bacillus subtilis var. niger; however, each laboratory has used their own unique experimental dry heat test system. The results of the cooperative study between the University of Minnesota and the U.S. Public Health Service, Phoenix Laboratory described in Progress Report 4 indicates that there can be differences in the results obtained using different test systems. We have developed several different systems for measuring dry heat resistance of microorganisms under different conditions. An important future objective of the University of Minnesota Planetary Quarantine program is to extend the environmental-condition capability of these different systems so there is an overlap.. We will carryout tests in both systems at the same environmental condition. The results will be used to identify those system parameters that are responsible for these differences and perhaps at the same time to illustrate heretofore unknown variables that affect the dry heat destruction rate of microorganisms.

I. J. Pflug

SURVIVAL OF MICROBIAL SPORES UNDER SEVERAL TEMPERATURE AND HUMIDITY CONDITIONS

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INTRODUCTION

In previous progress reports we have described and reported on experiments carried out to measure the survival rates of Bacillus subtilis var. niger spores which were suspended in solutions of sucrose and glycerol which had been adjusted to water activities of .85, .90 and .99, and heated at temperatures of 45, 60, 75 and 90°C. The final report of these experiments will be presented in Progress Report #8.

This report is concerned with activities carried out to determine the effect of the temperature and relative humidity of the surrounding atmosphere on the survival of Bacillus subtilis var. niger spores. These tests were performed in the controlled environmental chamber described in Progress Report #6 at temperatures of 75 and 90°C and at relative humidities of 15, 35, 55, and 75%.

OBJECTIVE

The objective of this task is to determine the effect of the relative humidity of the atmosphere at temperatures of 75 and 90°C on the survival of spores deposited on surfaces.

EXPERIMENTS PERFORMED AND RESULTS OBTAINED

The test procedures used in performing these experiments were described in Progress Report #6. All tests were conducted using spores of Bacillus subtilis var. niger deposited on stainless steel strips. Initially, three replicate strips were heated per time interval. In some instances there was an unexplained variation in the survival of spores on the replicate strips; therefore the number of replicates per heating time was increased to five. Table 1.1 summarizes the tests completed to date.

Four tests were performed at 90°C and 75% relative humidity. The D-values and the dates the tests were performed are listed in Table 1.1; the survivor curves are shown graphically in Figures 1.1-1.4. The differences between the survivor curves obtained early in 1971 (Figures 1.1 and 1.2) and those obtained late in 1971 (Figures 1.3 and 1.4) are unexplained. Additional tests will be

conducted in an effort to determine the cause for these variations in results. In Figure 1.5 are shown representative survivor curves for 15, 35, 55 and 75% relative humidity at 90°C (survivor curves for the 15 and 35% relative humidities were reported in Progress Report #6.) These results are consistent with the results from tests conducted at 22, 45 and 60°C which were presented in Progress Reports 3 and 4.

Survivor curves are presented in Figure 1.6 for tests conducted at 55 and 75% relative humidity at 75°C. It is interesting to note that at 55% relative humidity there is a difference in the shape of the two survivor curves; tests were carried out in May and September. The difference in shape parallels the difference in shape found at 90°C (Figures 1.1 and 1.2 are a different shape than Figures 1.3 and 1.4).

At both 75 and 90°C the time required to produce a specific reduction in the number of surviving organisms increased with decreasing relative humidity. The data at 75 and 90°C are summarized in Figure 1.7 where the time for a two-log (99%) reduction in spore counts is plotted as a function of relative humidity. At 90°C the time for a two-log reduction in number of survivors is 8.5 hours at 75% relative humidity, 65 hours at 55% relative humidity, 190 hours at 35% relative humidity, 300 hours at 15% RH and 53 hours at 1.5% RH. Data at 1.5% RH have been included on the graph; these results were obtained using the hot plate-boat-planchet system of heating. At 75°C the time required for two-log reduction in viable spores is 91 hours with a change in relative humidity from 55 to 75%, the rate of reduction increased by a factor of 5 at 75°C and by a factor of 8 at 90°C.

In Figure 1.8 are plotted the D-values obtained at 55 and 75% relative humidity. The resulting z-values are 11°C at 75% RH and 17°C at 55% RH. Angelotti found z-values that ranged from about 20°C to 30°C for a range of water activities in epoxy plastic. In wet heat the z-value is about 10°C. Therefore, our data is consistent with other data and it all suggests that the z-value may uniformly increase as the relative humidity of the system decreases from 100%.

Table I.I

Summary of Survivor Tests Performed on *Bacillus subtilis* var. *niger* Spores in the Controlled Environmental Chamber

Exp #	Date	No. of Replicates	Temp.	RH	D-value in hrs.	95% CI
GS1063A	3-4-71	3	90°C	75%	5.7	4.6-7.5
1110A	4-20-71	3	"	"	4.3	3.5-5.8
1306A	11-2-71	5	"	"	1.8	1.6-2.1
1308A	11-4-71	5	"	"	1.4	1.2-1.6
1313A	11-9-71	5	"	55%	28.1	24.6-32.7
1320A	11-16-71	5	"	"	32.9	29.6-37.1
1012A	1-12-71	3	"	35%	76.2	64.6-93.2
1089A	3-30-71	3	"	"	65.1	59.4-72.1
1335A	12-1-71	5	"	"	61.8	56.3-68.4
0342D	12-8-70	3	"	15%	121.3	103.6-146.3
1236A	8-24-71	3	75°C	75%	60.6	46.1-88.7
1134A	5-14-71	3	"	55%	250.1	208.3-313
1274A	10-1-71	4	"	"	170.6	153.9-191.5

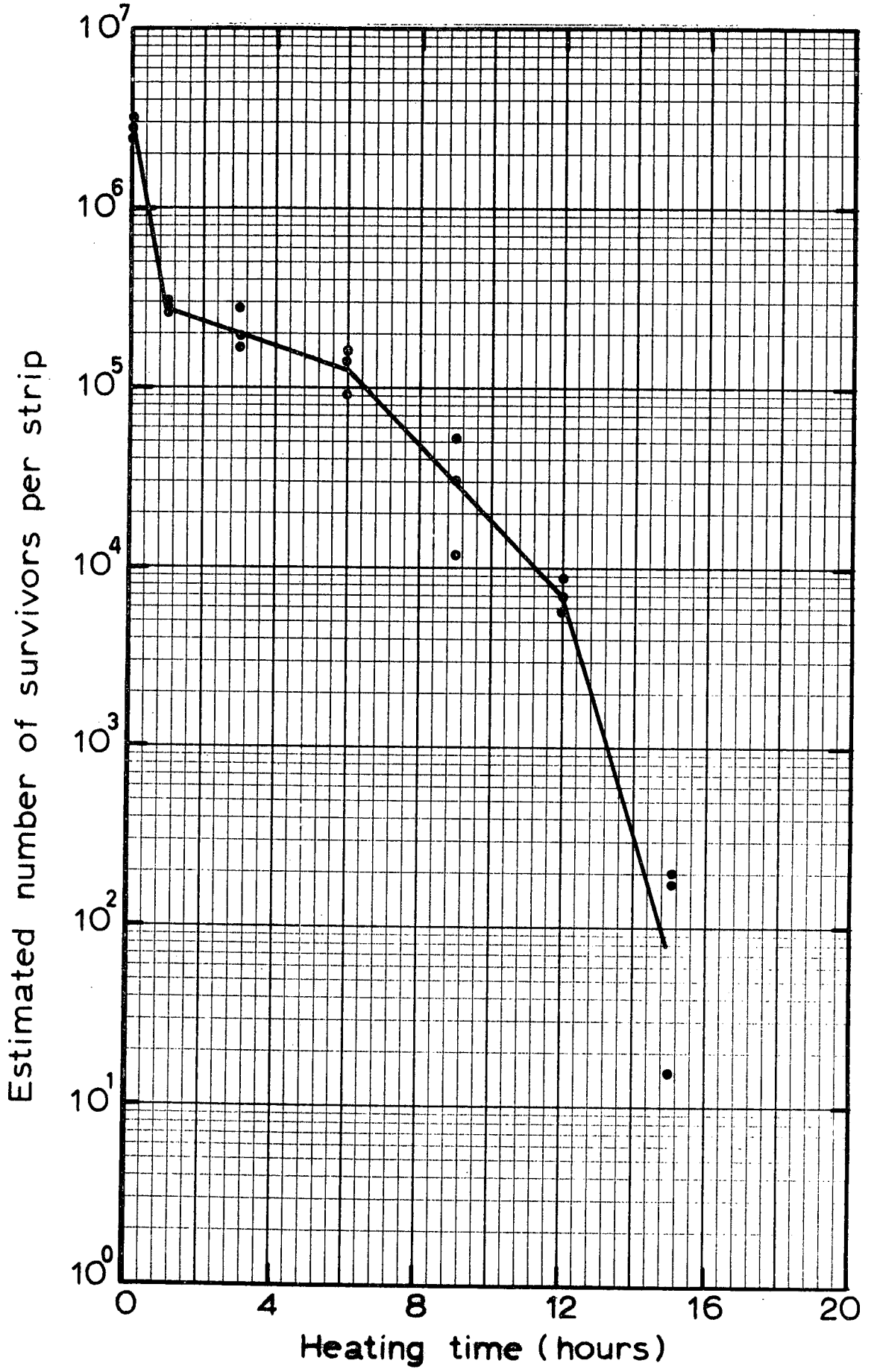

Figure I.1 - Survivor curve for Bacillis subtilis var. niger spores (AAOE) heated at 90°C and 75% RH, test GSIIIOA

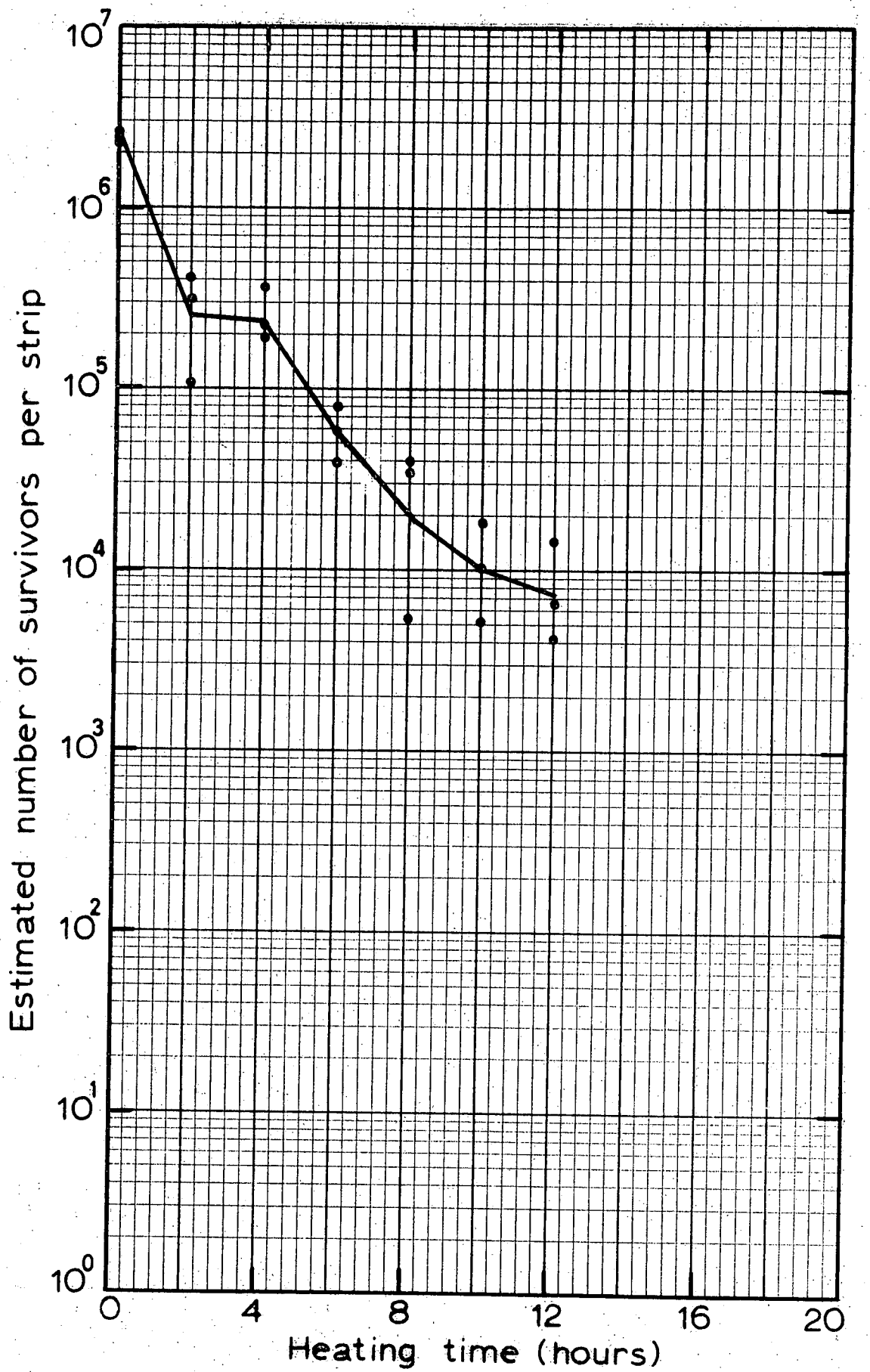

Figure 1.2 - Survivor curve for Bacillus subtilis var. niger spores (AAOE) heated at 90°C and 75% RH, test GS1063A

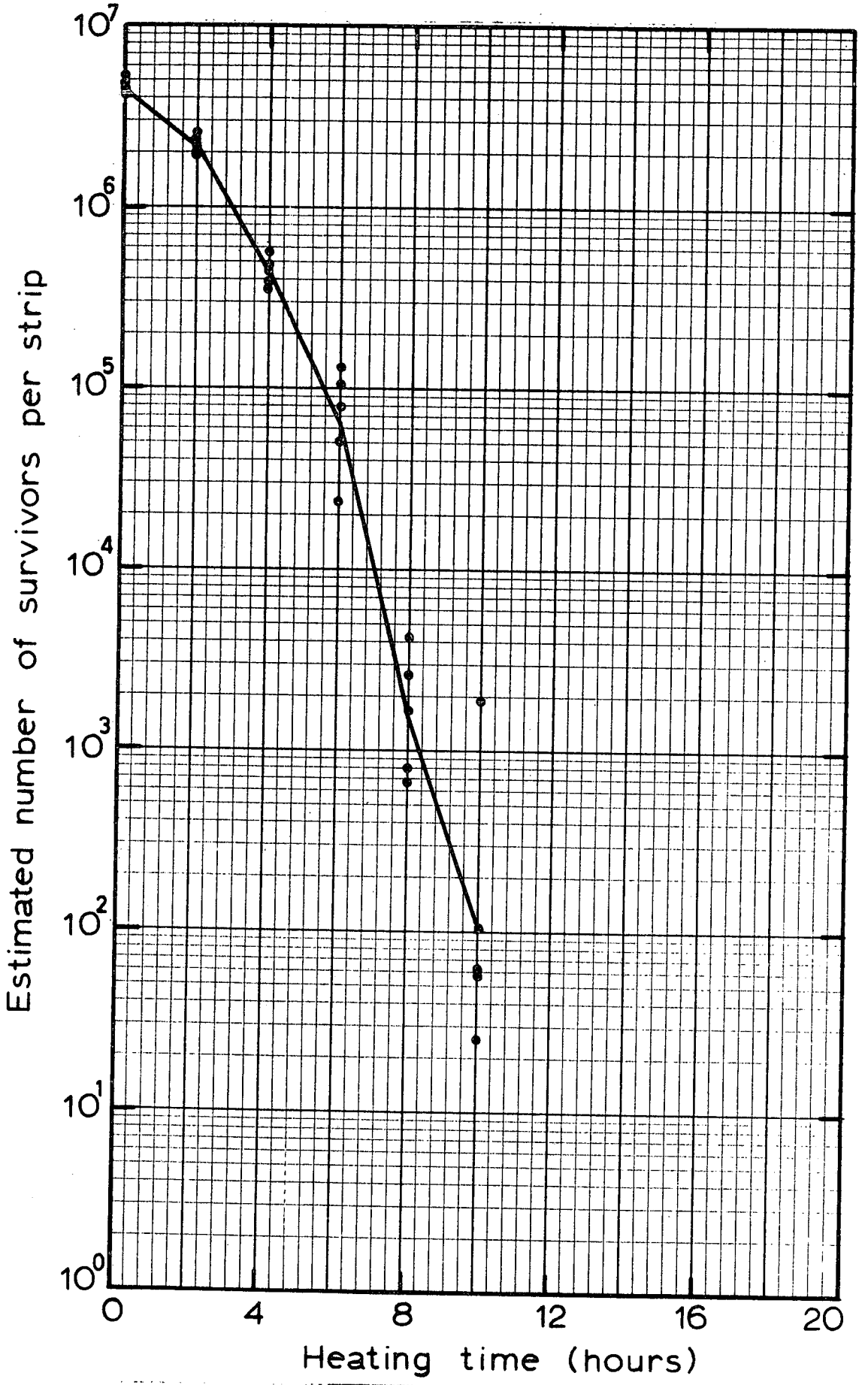

Figure 1.3 - Survivor curve for Bacillus subtilis var. niger spores (AAOE) heated at 90°C and 75% RH, test GS1306A

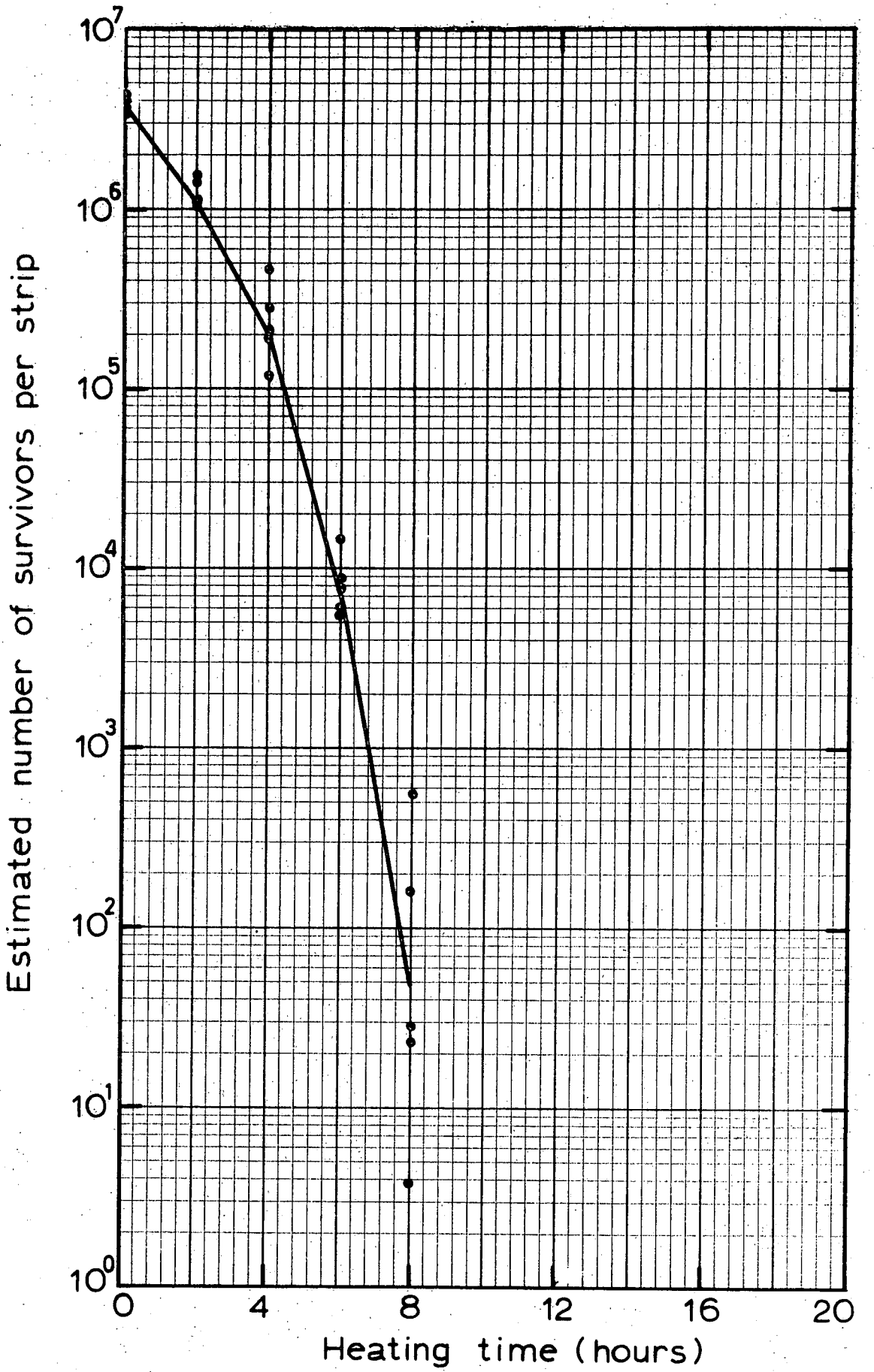

Figure 1.4 - Survivor curve for Bacillis subtilis var. niger spores (AAOE) heated at 90°C and 75% RH, test GS1308A

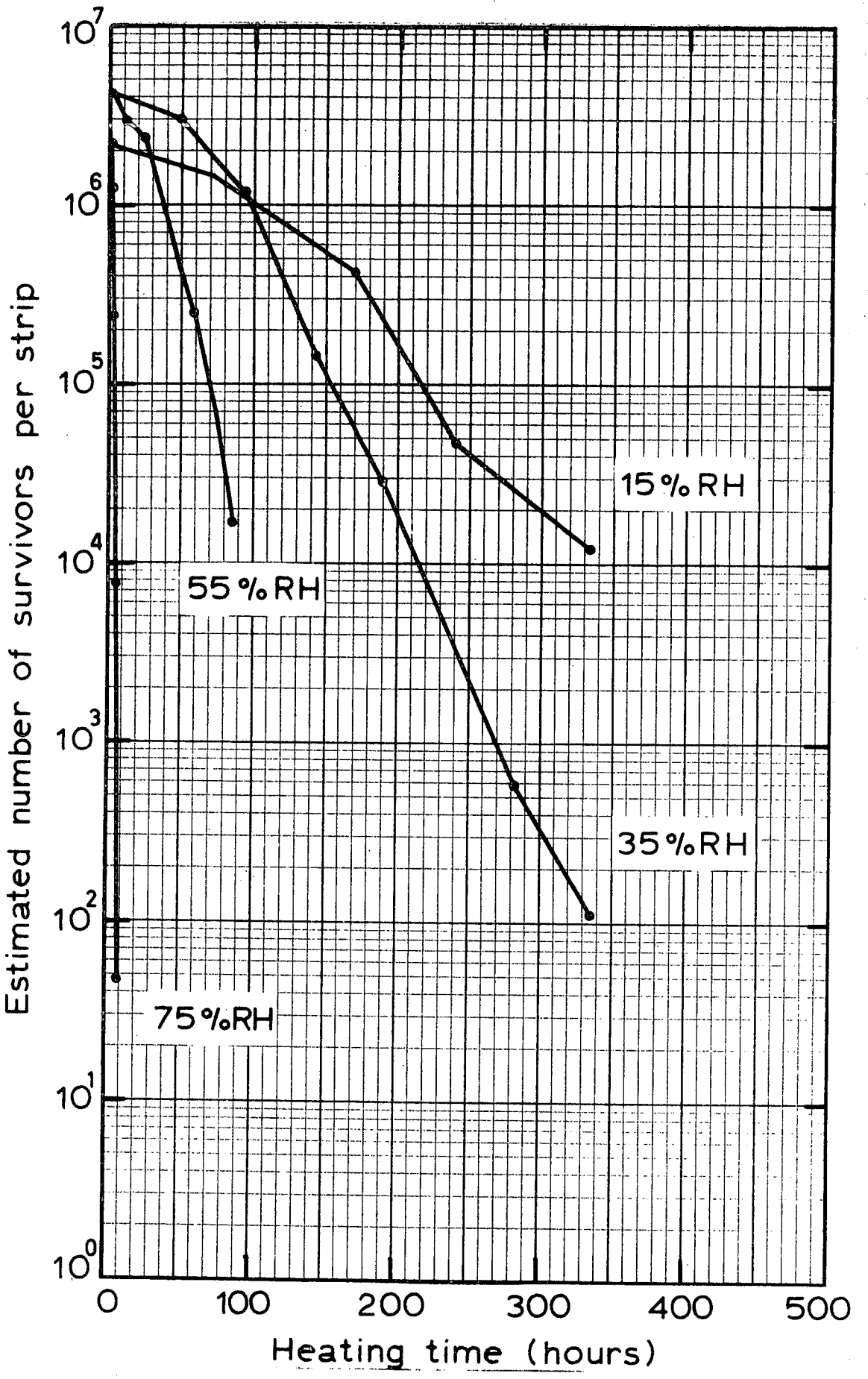

Figure 1.5 - Survivor curves obtained for Bacillus subtilis var. niger spores (AAOE) heated at 90°C and different RH tests GS1308A, GS1320A, GS1335A, and GS0342D

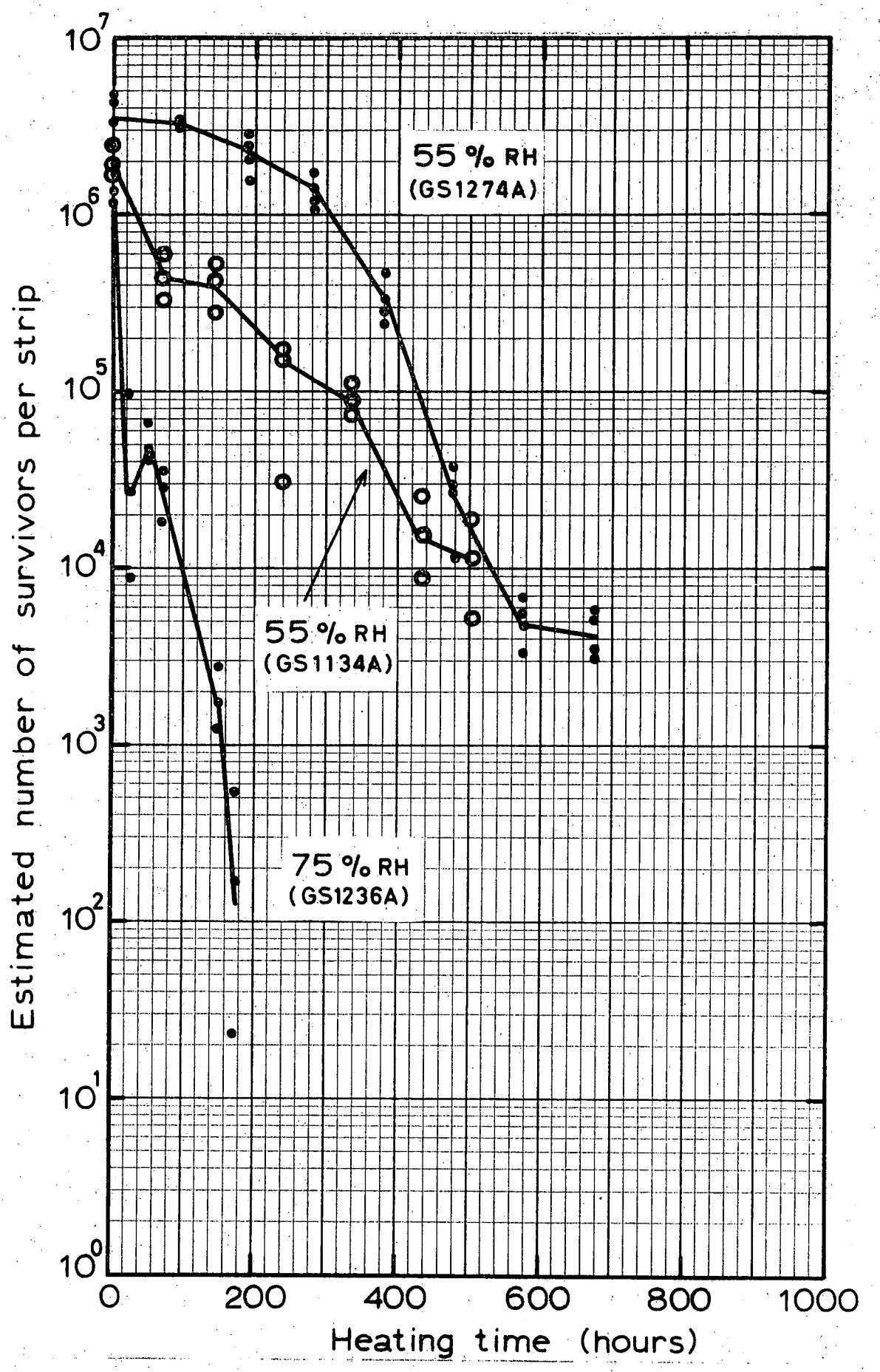

Figure 1.6 - Survivor curves for Bacillus subtilis var. niger spores (AAOE) heated at 55 and 75% RH at 75°C

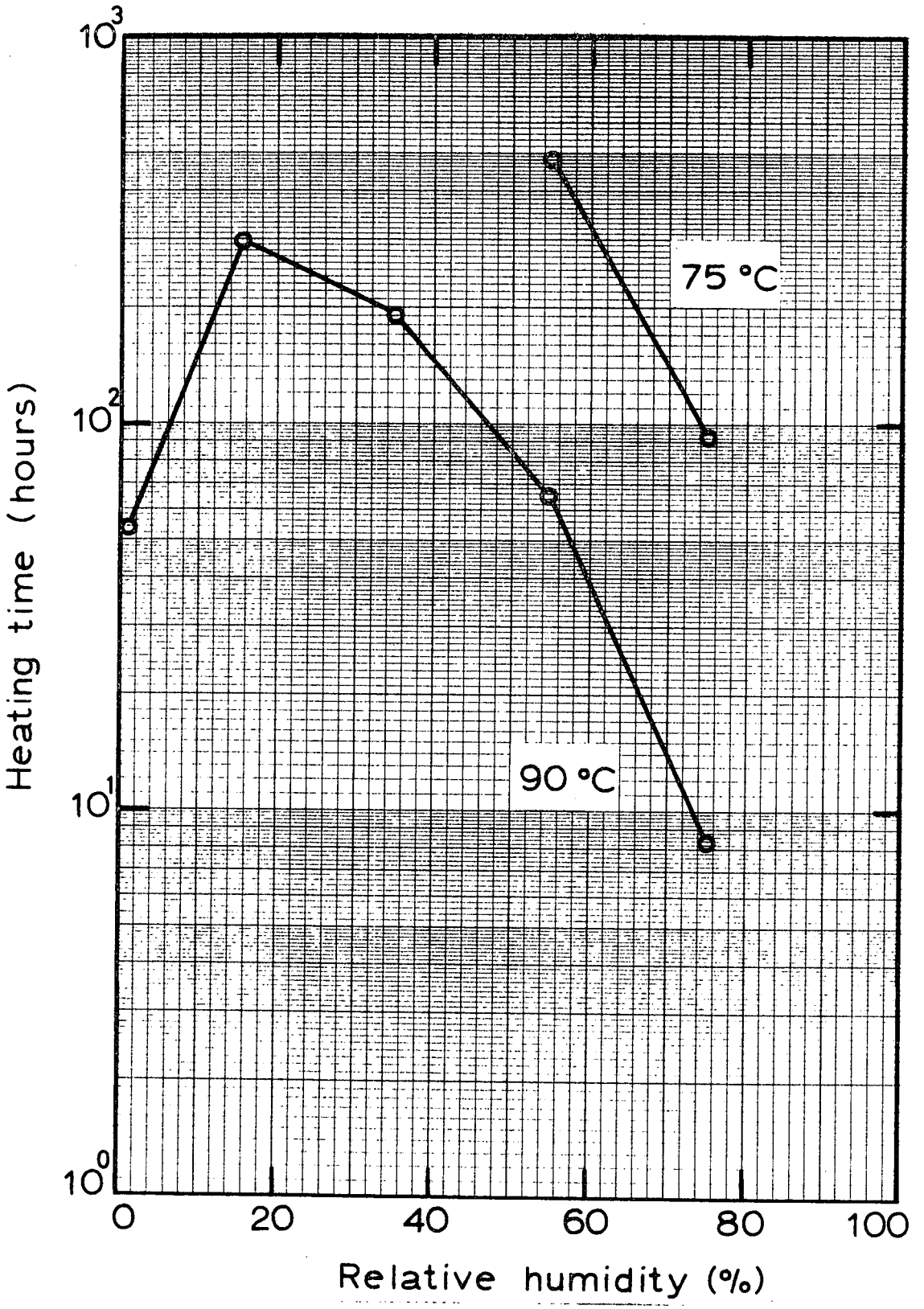

Figure 1.7 - Estimated time for a 99% reduction in the number of survivors at 75 and 90°C as a function of test relative humidities

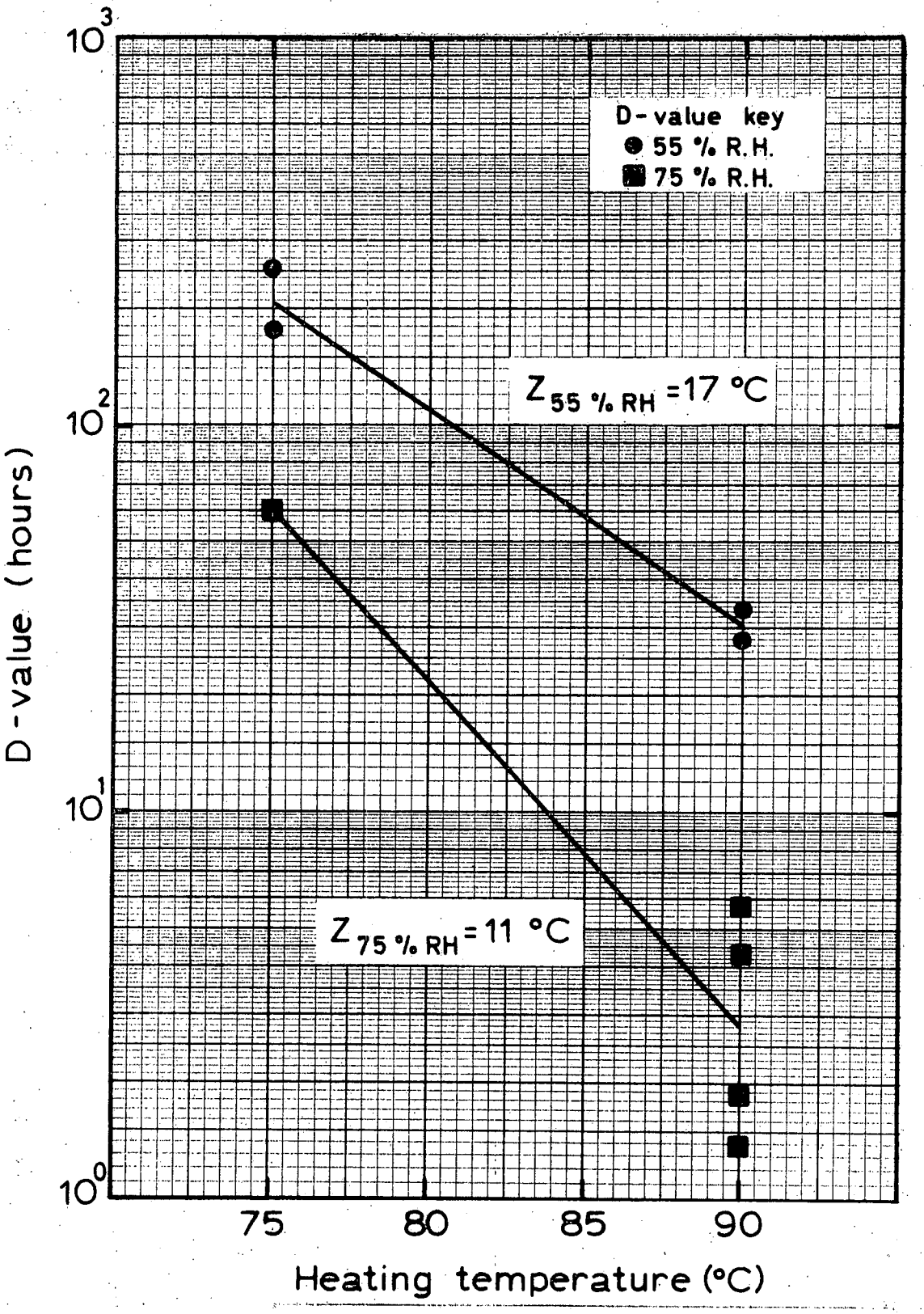

Figure I.8 - Thermal destruction curves obtained for Bacillus subtilis var. niger (AAOE) spores using the Hot Pack environmental control system

DRY HEAT DESTRUCTION RATES OF MICROORGANISMS ON SURFACES

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INTRODUCTION

The results of our preliminary investigation into the survival characteristics of microorganisms in soil when subjected to dry heat appeared in our sixth progress report. These results suggested a possible relationship between particle size and the number of surviving colony-forming units. During this reporting period we have carried out the following four different groups of experiments designed to elucidate the characteristics of microbial spores associated with soil particles: 1) the effect of particle size on the dry heat destruction of spores; 2) the survival of microorganisms conditioned in acidified and non-acidified water; 3) the survival of microorganisms in Cape Kennedy soil treated in the clean room and glove box; and 4) a comparison of the survival of microorganisms in Cape Kennedy soil with the survival of microorganisms in a Minnesota soil.

OBJECTIVE

The objective of these studies is to develop a better understanding of the resistance of microorganisms in soil to destruction by dry heat. We are specifically attempting to determine whether there is a relationship between the size of the soil particles and the dry heat resistance of the spores attached to or enclosed by these particles.

EXPERIMENTAL PROCEDURE

In all of the dry heat survival tests conducted on microorganisms associated with soil, the planchet-boat-hot plate system described in Progress Report #3 was used. The hot plate temperature was 125°C. All soil was suspended in 95% ethanol. The experimental design and procedure are reported in Appendix A. Both plate count and fraction negative assay methods were utilized. Our entire microbiological analysis procedure was carried out in a class 100 laminar flow clean room under an operational regime that offered minimum opportunities for contamination.

Some of the inoculated planchets were processed using the following plate count assay method:

1. The planchets were insonated for two minutes in 25 ml of buffered

distilled water.

2. Duplicate 10 ml aliquots were plated using Trypticase Soy Agar.
3. The plated aliquots from unheated soil were incubated for 48 hours at 32°C.
4. The plated aliquots from heated soil were incubated for one week at 32°C.

The remainder of the inoculated planchets were processed using the following fraction negative assay method:

1. The planchets were placed into 25 x 150 mm test tubes containing 10 ml of Trypticase Soy Broth plus .04% bromcresol purple and .005% alanine.
2. The tubes containing media and planchets were incubated at 32°C and were observed after 1, 2 and 3 weeks.
3. Those samples with questionable growth were subcultured on TSA, TGY and supplemented TAM agar plates. If no growth was observed, the results were declared negative.

In tests SMI201A, B, and C, the negative samples were reincubated at 55°C for two additional weeks in an attempt to detect the presence of thermophilic organisms. No additional positive samples were found.

In our analysis of the data from tests where the number of survivors was determined by plate count procedures, we used plate count numbers as low as one colony per plate. When we used three planchets per boat and two boats per heating time, twelve plate count values were available for each heating time. If there was one colony on each of the twelve plates, the estimated number of survivors per planchet was 2.5. However, if there was only one colony on one of the twelve plates, then the calculated number of survivors per planchet was 0.2. In our results we are utilizing all data including those data where only one of the twelve plates has one colony.

In the fraction negative studies we utilized either two or three boats per time and four or five planchets per boat. In all cases the minimum number of planchets per heating time was ten; twelve planchets per heating time were used in most of the fraction negative work. The fraction negative results are quite consistent with the plate count results; however, when we used the fraction negative method it was possible to explore the longer times more efficiently than when the plate count method was used.

Effect of Particle Size on the Dry Heat Destruction Rate of Microbial Spores Associated with These Soil Particles

In Progress Report #6 we described preliminary dry heat resistance studies in which we evaluated Cape Kennedy soil that had been separated into different particle sizes by a sedimentation technique. These preliminary experiments showed a trend for the organisms associated with large particles to be more resistant to dry heat

than those organisms associated with small particles; however, the results were inconclusive.

We have developed what we believe is an improved method of particle size separation incorporating both sedimentation and filtration procedures (see Appendix C). The experiments described below were performed using Cape Kennedy soil which had been separated into different particle sizes using this new procedure.

We carried out two different separations and each separation resulted in three particle sizes: >10-<43; >3-<10; and <3 microns. Each fraction size sample was titered and the concentration adjusted so that each sample would have approximately the same number of colony-forming units per unit volume as determined by the plate count method. If after two adjustments the obtained titers were relatively close or favored the smaller fractions, no further adjustments were made. It should be noted that there were problems encountered in titering all fractions since the growth of some colonies spread widely during the 48-hour incubation period. Spreading colonies were not observed on plates containing heated soil samples.

Duplicate dry heat tests at 125°C were conducted on each separation. Each test used both plate count and fraction negative assay procedures. Deposition and equilibration of the soil suspension, the heating and cooling of the boats and the processing of the planchets were carried out in the laminar flow clean room operated at approximately 23°C and 50% RH.

A summary of the particle size experiments is shown in Table 2.1.

Table 2.1

Summary of Particle Size Soil Experiments

Test number	Spore code	Particle size	Number of units	
			Plate counts	Fraction negative
SMI187A[1]	WAEIB	>10μ<43μ	2 boats/time	2 boats/time
B	C	>3μ<10μ	3 planchets/boat	5 planchets/boat
C	D	<3μ		
SMI201A[1]	WAEIB	>10μ<43μ	3 boats/time	3 boats/time
B	C	>3μ<10μ	3 planchets/boat	5 planchets/boat
C	D	<3μ		
SMI194A[2]	WACIB	>10μ<43μ	3 boats/time	3 boats/time
B	C	>3μ<10μ	2 planchets/boat	4 planchets/boat
C	D	<3μ		
SMI208A[2]	WACIB	>10μ<43μ	3 boats/time	3 boats/time
B	C	>3μ<10μ	2 planchets/boat	4 planchets/boat
C	D	<3μ		

[1]separation 1

[2]separation 2

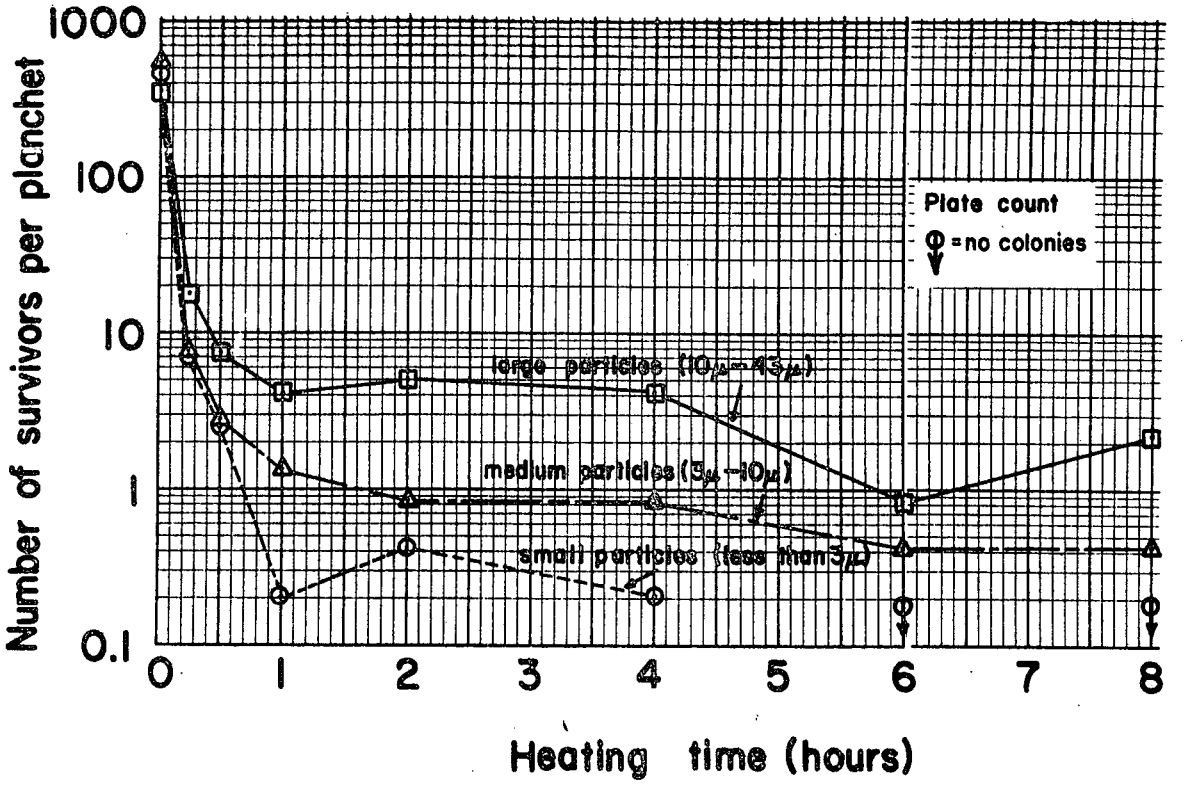

Figure 2.1 - Effect of particle size Test SMI187, 1st separation, 1st replication

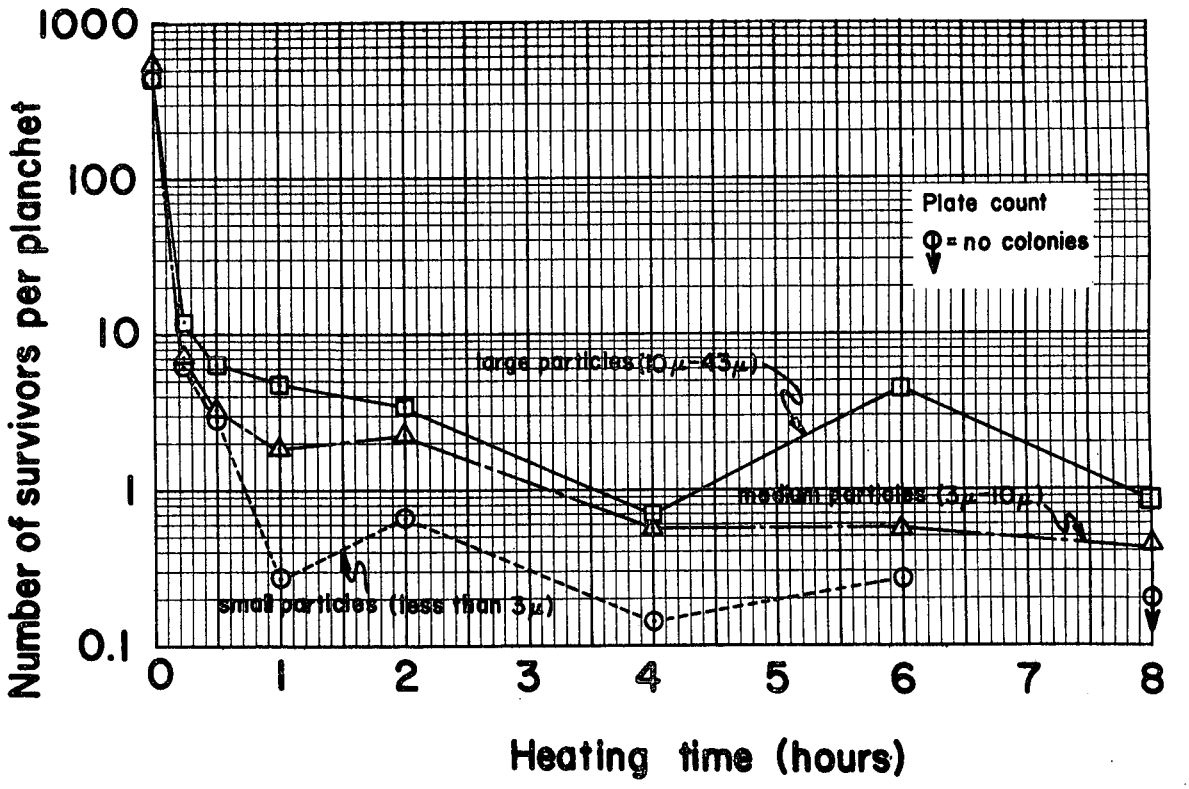

Figure 2.2 - Effect of particle size Test SMI201, 1st separation, 2nd replication

In the plate-count survivor curve test results (shown in Figures 2.1, 2.2, 2.3 and 2.4) we observed that there was a consistently larger number of surviving organisms associated with the large particles than with the medium-size particles. This same type of relationship existed between the medium and small-size particles; there was a larger number of surviving organisms associated with the medium size particles than with the small-size particles. The difference appeared at the shortest heating time of 1/4 hours and reached a more or less stable condition at 1/2 or one hour. Following heating times of from 1 to 8 hours, it appeared that a stable pattern developed where the relative number of survivors was highest for the largest particles and lowest for the smallest particles. We found this consistency in all four experiments which consisted of two different separations and two tests for each separation. There was some variation but we feel that considering all of the manipulations required, the consistency of these data substantiates the fact that the differences in survival associated with particle size are real.

Using the fraction negative method, we explored heating times of up to 48 hours. The results of these tests are shown in Figures 2.5, 2.6, 2.7 and 2.8. There were survivors associated with the large particles in all tests at 32 hours and in one test at 40 hours. For the small particles we found no survival at 4 hours for one experiment, 6 hours for one experiment and 8 hours for one experiment. However, in the fourth experiment there were survivors at both 16 and 32 hours. In summary, about 1% of the viable population associated with large particles survived one hour of heating whereas approximately .04% of the viable organisms associated with the small particles survived one hour of heating.

Discussion

The fact that we found a less-resistant population associated with the small particles suggests that we have either a more resistant fraction associated with the large particles, or, if the same type and number of organisms are associated with all particles, then the large particle affords protection for the organisms during the initial heating period. The data suggest that all organisms that are unable to survive one hour at 125°C will die at about the same rate regardless of whether they are associated with small, medium or large particles.

The initial viable count was approximately the same for all of the particle sizes that we evaluated. The number of survivors that we observed was different in that the large particles had a larger number of survivors than the small sizes.

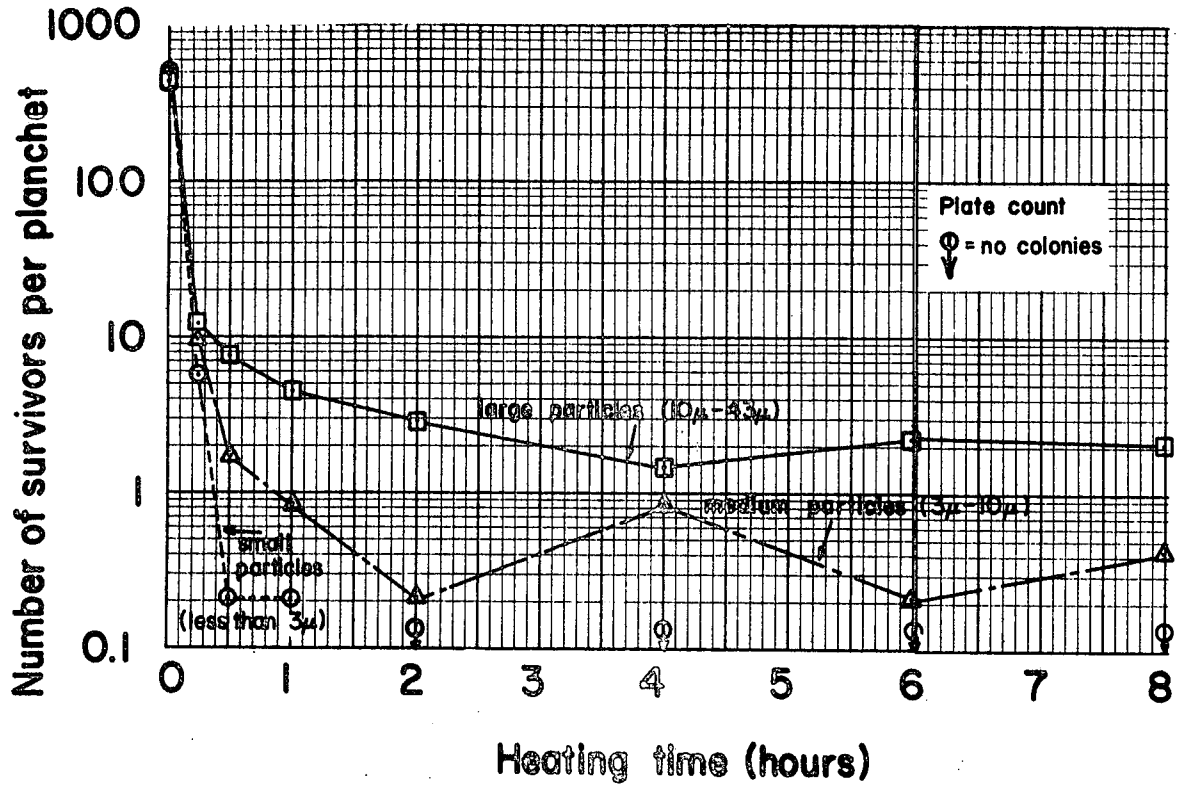

Figure 2.3 - Effect of particle size - Test SMI194, 2nd separation, 1st replication

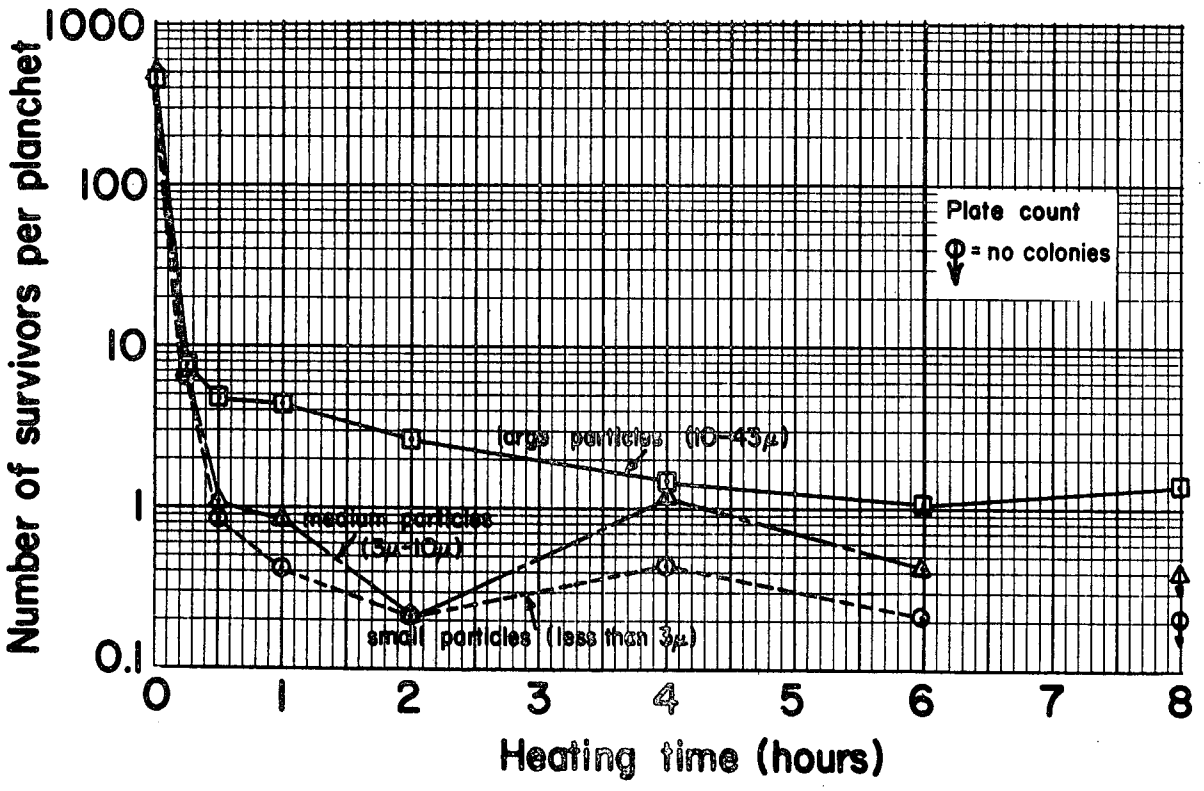

Figure 2.4 - Effect of particle size - Test SMI208, 2nd separation, 2nd replication

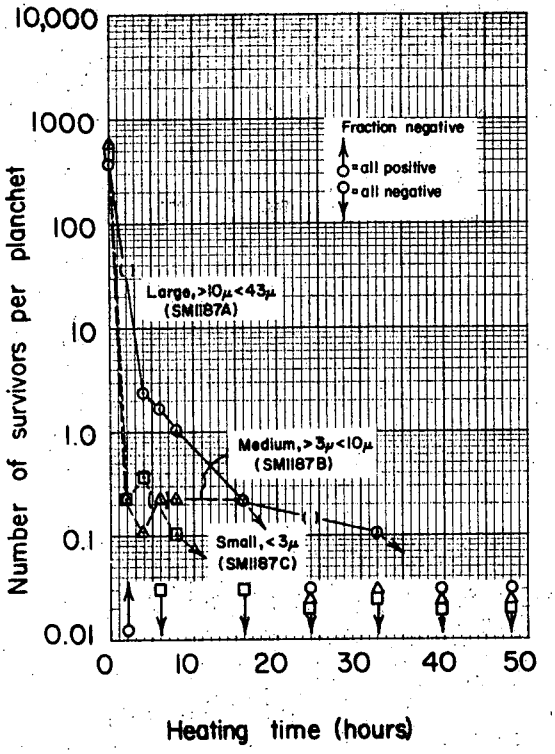

Figure 2.5 - Effect of particle size - 1st separation, 1st replication

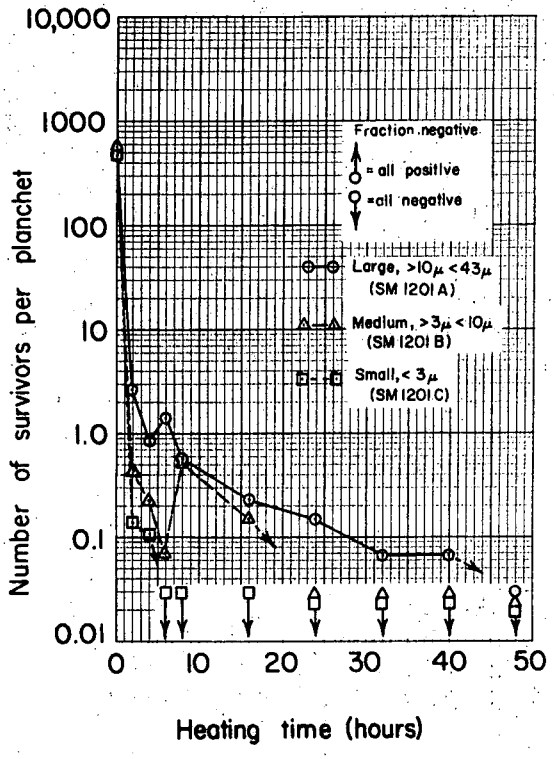

Figure 2.6 - Effect of particle size - 1st separation, 2nd replication

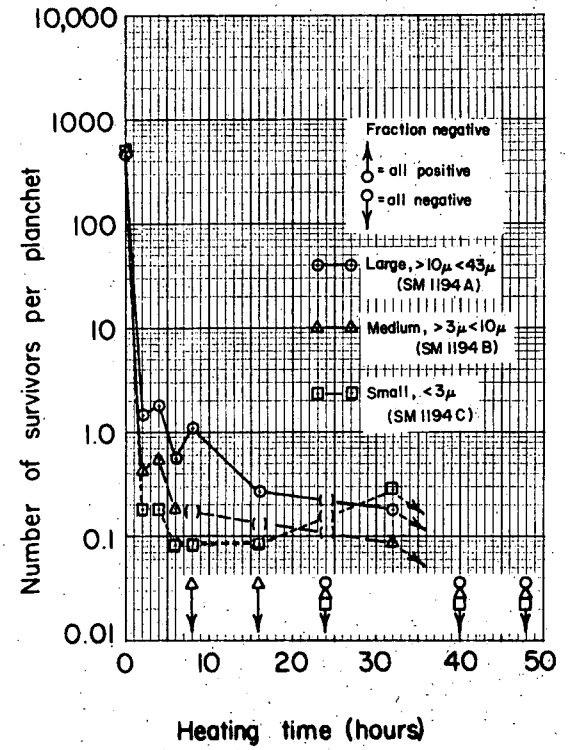

Figure 2.7 - Effect of particle size - 2nd separation, 1st replication

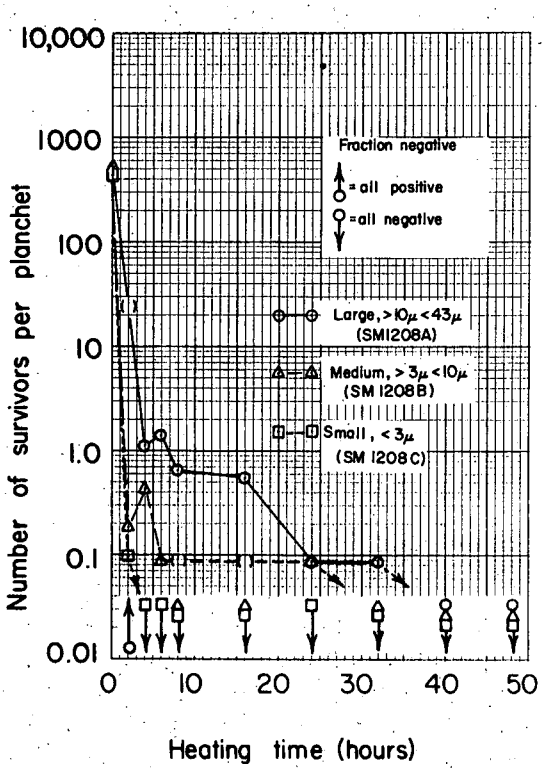

Figure 2.8 - Effect of particle size - 2nd separation, 2nd replication

The question can be raised regarding whether we were counting the same initial number of organisms in each case. Since we were dealing with microorganisms associated with soil particles it is possible that there were more spores associated with each of the larger particles than with each small particle. We believe that it is realistic to assume that in many cases there was more than one spore associated with a soil particle and that not all of these particles were broken up in the insonation process carried out prior to dilution and plating.

The work carried out here is relative and relevant to actual spacecraft conditions since we measured initial numbers in the same way that initial numbers will be assayed on the spacecraft. Our organism counts associated with large, medium and small particles are representative of organism counts that will be found on the spacecraft for the same range of particle sizes.

Comparison of Survival of Microorganisms Conditioned in Acidified and Non-acidified Water

In the previously described study we found that the microbial spores associated with the larger particles survived a longer dry heat treatment than the spores associated with the smaller particles. If we assume that the spores are randomly distributed with regard to dry heat resistance on and in soil particles of a similar size, then it would seem logical that the difference in dry heat resistance observed is due to some special relationship of the spore and the soil.

It was theorized that the Cape Kennedy soil particles were a mixture of water soluble crystals and water insoluble particles. The spores would be inside water soluble crystals or inside soil particles where both water soluble crystals and insoluble particles are cemented together by water soluble materials. If this was, in fact, the situation then we would expect the dry heat resistance of spores associated with large particles to decrease after a water soaking treatment.

Two sets of experiments were carried out to determine if the survival of microorganisms associated with soil particles is altered by holding the soil in water for a few days.

Procedure

A sample of Cape Kennedy soil containing particles ranging in size from 10 to 30 microns, was suspended in ethanol and then divided into 10 ml aliquots. The following conditioning procedure was carried out at two different times; two aliquots of this suspension were used for each time.

Two 10 ml samples were centrifuged and the excess ethanol decanted. The two samples were then placed in a vacuum dessicator at a pressure of about 5 in. Hg abs.

for 96 hours to remove the remaining ethanol. Fifty ml of sterile distilled water, which had been adjusted to a pH of 5.5 with 0.1 normal (N) hydrochloric acid (HCl) and which contained 0.012% bromcresol purple (BCP) as a pH indicator, were mixed with the two samples. The pH of a conditioning soil sample was determined by comparing the color of the sample with a set of BCP pH color standards.

When soil was added to the water the pH increased to above 7.0 as indicated by the color standard and about 8.0 as determined using a pH meter. In one sample, the pH was adjusted periodically with 0.1 N HCl to try to maintain a pH of 5.5. In the other sample the buffering action of the soil was allowed to determine the pH of the suspension. After 72 hours of agitation on a magnetic stir plate at 4°C, the water was removed from the sample using a .45 micron screen filter. The pH of the conditioning water was measured and recorded. The filters were allowed to air dry for 18 hours and the soil was removed by insonation in 95% ethanol. The volume of each soil-ethanol suspension was made up to 10 ml.

A total of 36 drops of 0.1 N HCl was added during the first conditioning and the final pH of the water was 6.8. During the second conditioning, 55 drops of 0.1 N HCl were added and the final pH of the water was 6.2. The pH's of the untreated waters were 7.9 and 8.0 respectively.

The soil-ethanol samples resulting from the first conditioning treatment were subjected to heat resistance experiments at 125°C in the clean room. The soil-ethanol samples resulting from the second conditioning treatment along with a control that was not subjected to the agitated water treatment were tested in both the clean room and the dry glove box. The experimental protocol outlined in Appendix A was followed using three planchets per boat, two boats per heating period for the plate counts, and four planchets per boat, three boats per heating period for the fraction negative tests. Experimental conditions of the tests performed are given in Table 2.2.

Table 2.2

Experiments Conducted Using Acidified Water

Run	Conditioning environment	Spore code	Test Number	Test Environment	Test Number	Test Environment
1	acidified water	WAH1A	SM1264A	clean room[1]	None	
	water	B	B	" "		
2	acidified water	WAH1C	SM1271A	clean room[1]	SM1285A	glove box[2]
	water	D	B	" "	B	" "
	control	E	C	" "	C	" "

[1]At test temperature (125°C) the RH was 0.57% in the clean room

[2]At test temperature (125°C) the RH was 0.00013% in the glove box

Results of these tests are shown graphically in Figures 2.9 - 2.14.

The survivor curves obtained from tests conducted in the clean room were characterized by a rapid reduction in the number of viable particles within 30 minutes followed by a long period of time during which there was very little reduction in count. During the first 30 minutes the number of survivors was reduced from about 3000 to 30. After 8 hours at 125°C we found between 1 and 10 survivors. Survivors were detected after as long as 40 hours of heating at 125°C.

In general it appeared that survival characteristics obtained in the glove box were similar to those obtained in the clean room. The estimated number of survivors was reduced by two or three logs within 30 minutes (somewhat faster than obtained in the clean room) followed by a very slow reduction. The average number of survivors at 8 hours was between 0.5 and 4 per planchet. Viable particles were observed after 50 hours on the 125°C hot plate. In all cases, the samples conditioned in water had fewer survivors than the samples conditioned in acidified water. There was very little difference between the control and the samples conditioned in acidified water.

The differences observed in these experiments can be explained on the basis of the solubility of soil material in water at different pH levels. This of course suggests that the spores were protected by the soil.

Comparison of the Survival of Microorganisms in Cape Kennedy Soil Treated in the Clean Room and Glove Box

Previous studies on the dry heat resistance of Bacillus subtilis var. niger spores have shown that D-values for these spores are 2 to 3 times greater in the clean room environment than in the dry glove box environment.

The same large particles (WAIC,>10-<43μ) were heated both in the glove box and in the clean room and spore survival under the two conditions was compared. The tests performed are summarized in Table 2.3.

Table 2.3

Summary of Experiments on Cape Kennedy Soil Treated in the Clean Room and Glove Box

Test No.	Spore Code	Particle size	Number of units	
			Plate count	Fraction negative
SMI201[1]	WAEIB	>10-<43μ	3 boats/time 3 planchets/boat	3 boats/time 5 planchets/boat
SMI244[2]	WAEIB	>10-<43μ	3 boats/time 3 planchets/boat	3 boats/time 4 planchets/boat

[1]At test temperature (125°C) the RH was 0.57% in the clean room

[2]At test temperature (125°C) the RH was 0.00030% in the glove box

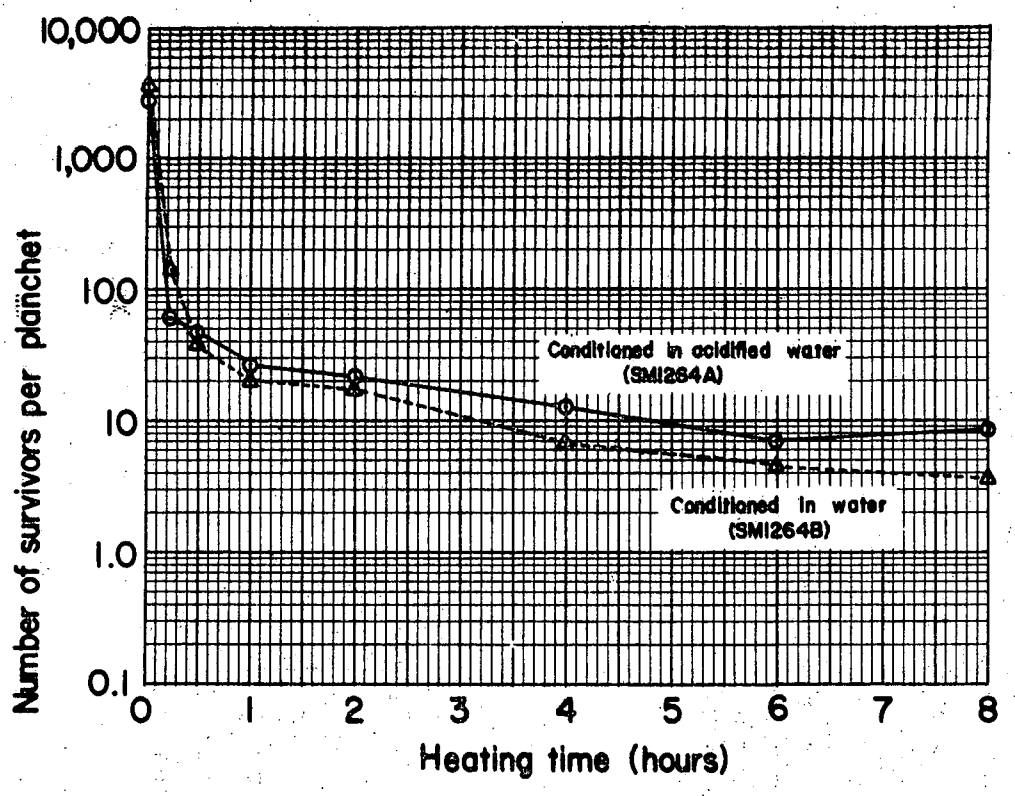

Figure 2.9 - Tests to determine the effect of holding soil in water on microbial survival

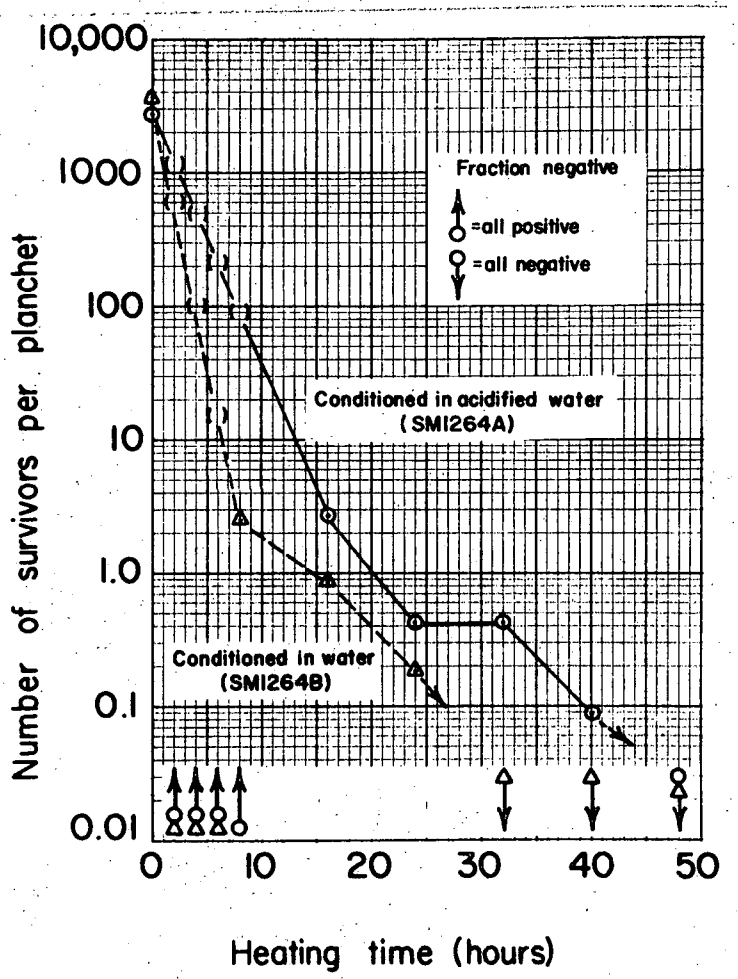

Figure 2.10 - Tests to determine the effect of holding soil in water on microbial survival

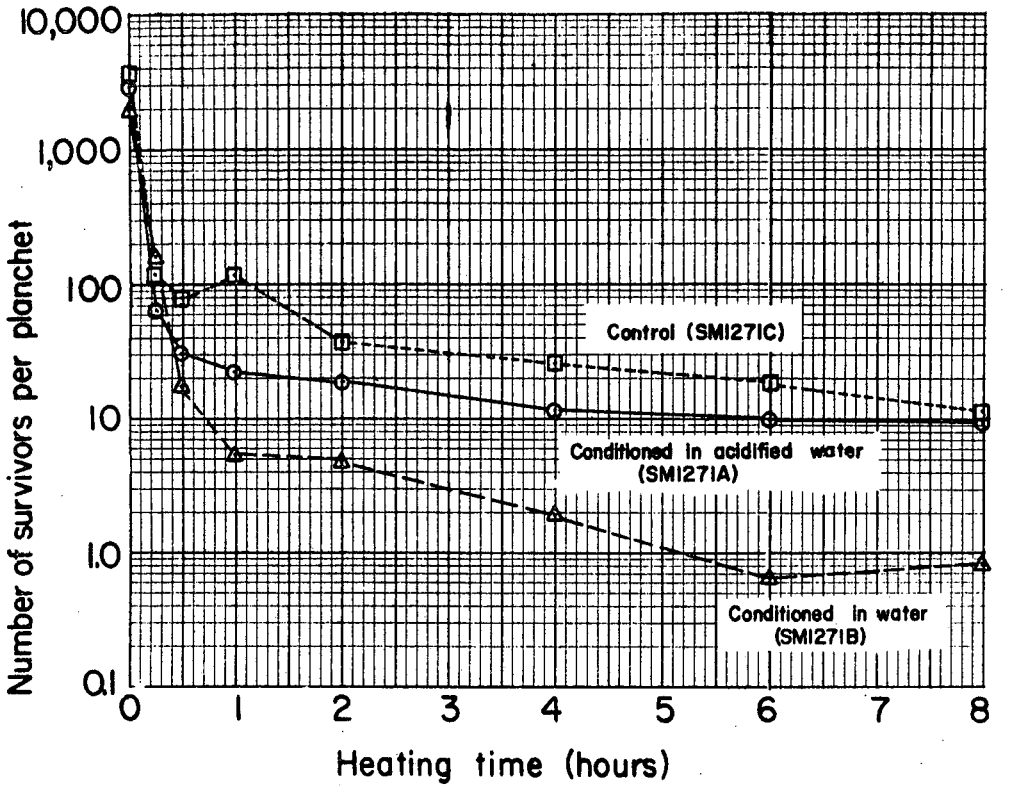

Figure 2.11 - Tests to determine the effect of holding soil in water on microbial survival

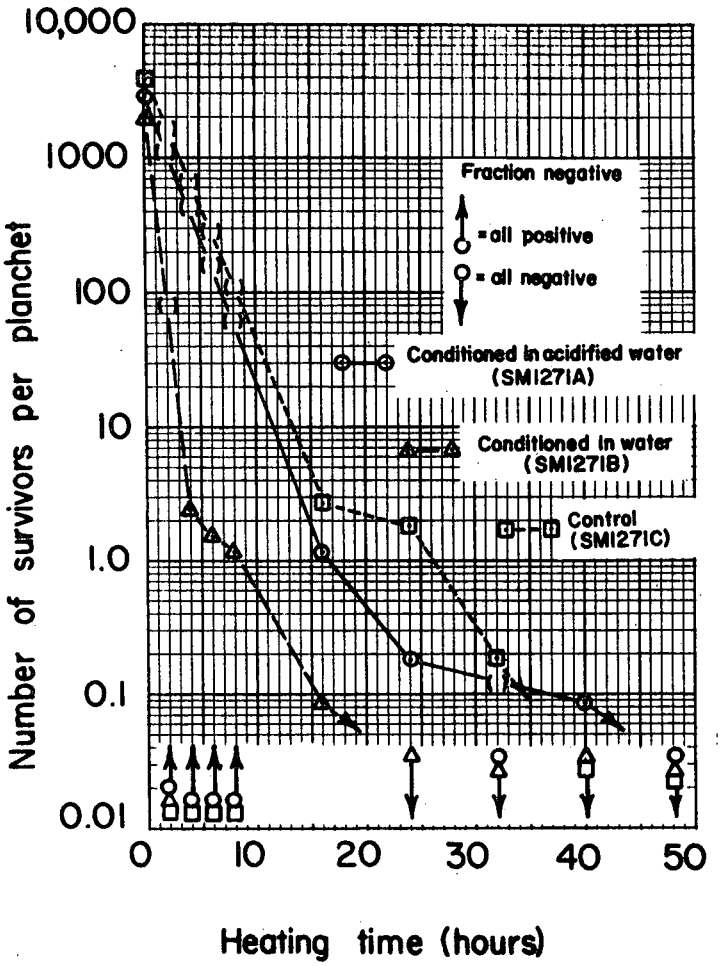

Figure 2.12 - Tests to determine the effect of holding soil in water on microbial survival

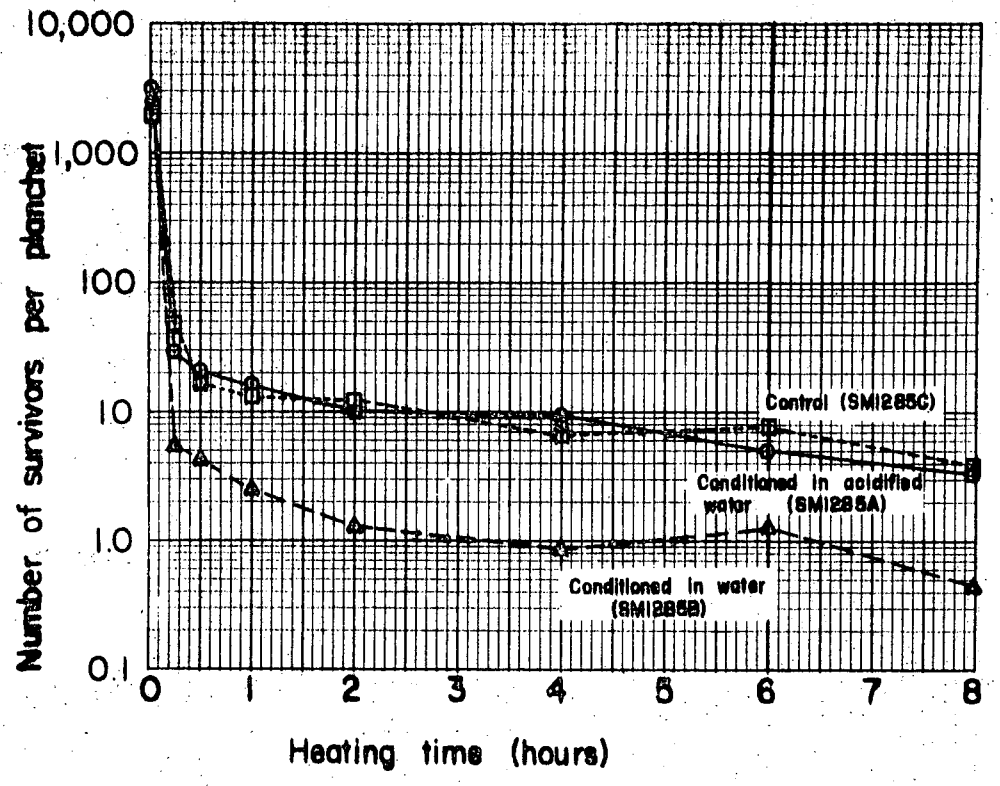

Figure 2.13 - Tests to determine the effect of holding soil in water on microbial survival

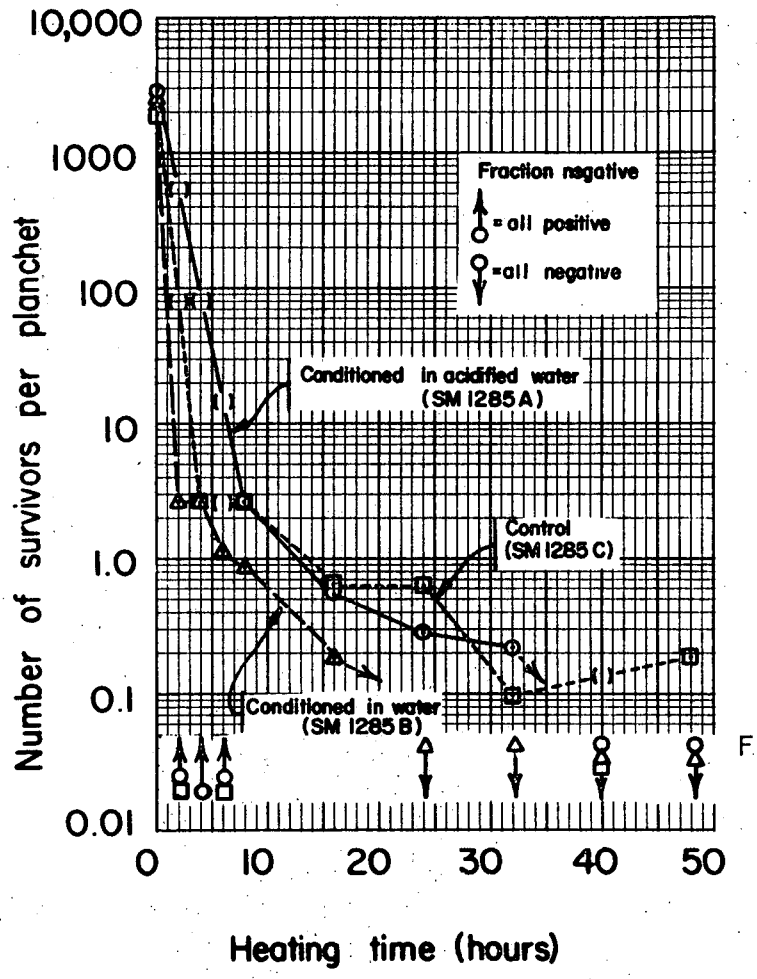

Figure 2.14 - Tests to determine the effect of holding soil in water on microbial survival

The results of these experiments are presented in Figures 2.15 and 2.16. There was essentially no difference in the number of survivors for heating times through 8 hours. However, for heating times that exceeded 8 hours, survivors were observed more often for samples heated in the clean room than for samples heated in the glove box. These results were somewhat surprising in view of the results obtained in previous studies. One possible explanation is that the soil protected the spore from dessication in the glove box during early heating times. Prolonged heating periods will cause a significantly greater reduction in the water content of the spores in the glove box which in turn will increase the spore death rate. The net result is relatively fewer survivors in the glove box following heating times longer than 8 hours.

Comparison of the Survival of Microorganisms in a Cape Kennedy Soil with the Survival of Microorganisms in a Minnesota Soil

A sample of soil was obtained from the Dinkytown area in southeast Minneapolis and the survival of microorganisms in this soil was compared with survival in the Cape Kennedy soil sample. Results of these tests are shown in Figures 2.17 and 2.18. Both soils show the characteristic initial rapid die-off and subsequent plateau. In the plateau portion of the curve the number of survivors for the Cape Kennedy soil (WAIIA) was ten times greater than for the Dinkytown soil (XFAIK). The fraction negative results indicate that through about 24 hours, the Dinkytown soil had a reduced number of survivors; however, the last survival time was 32 hours for the Dinkytown soil and 40 hours for the Cape Kennedy soil. This seems to suggest that these two soils were nearly equal in number of very resistant spores.

On two different occasions, dry heat tests at 125°C using both Cape Kennedy and Dinkytown soil were conducted in the clean room. Results of these tests are given in Figure 2.19. In this graph we have plotted the percent survivors as a function of heating time as a convenient method of getting all of the data on the same graph and normalizing the N_o point.

The general shape of all of the soil survivor curves is similar; it is the destruction rates within the first 30 minutes that appear to be different. The earlier tests (December, 1970) showed an initial lower destruction rate than the later tests (October, 1971). The survivor curves of the respective spores tended to parallel each other after the first 30 minutes.

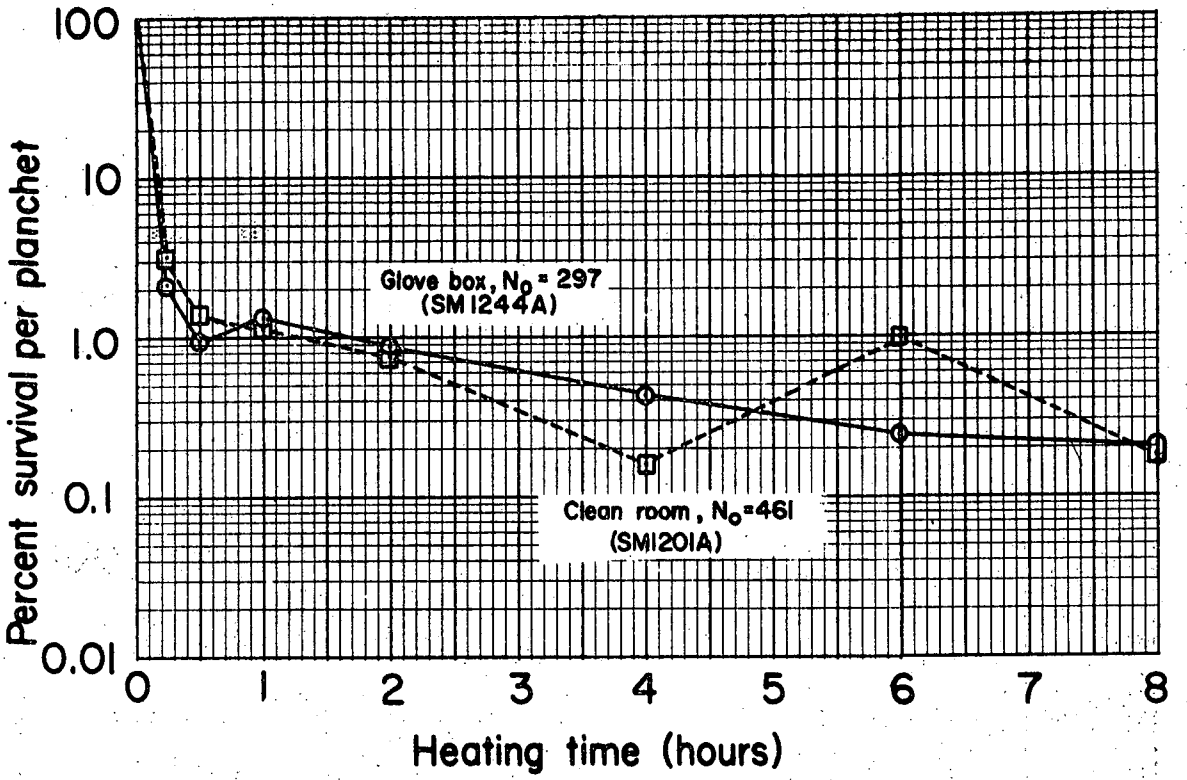

Figure 2.15 - Survival characteristics of organisms in soil heated in clean room versus soil heated in the glove box; survivor curve determined by plate count method

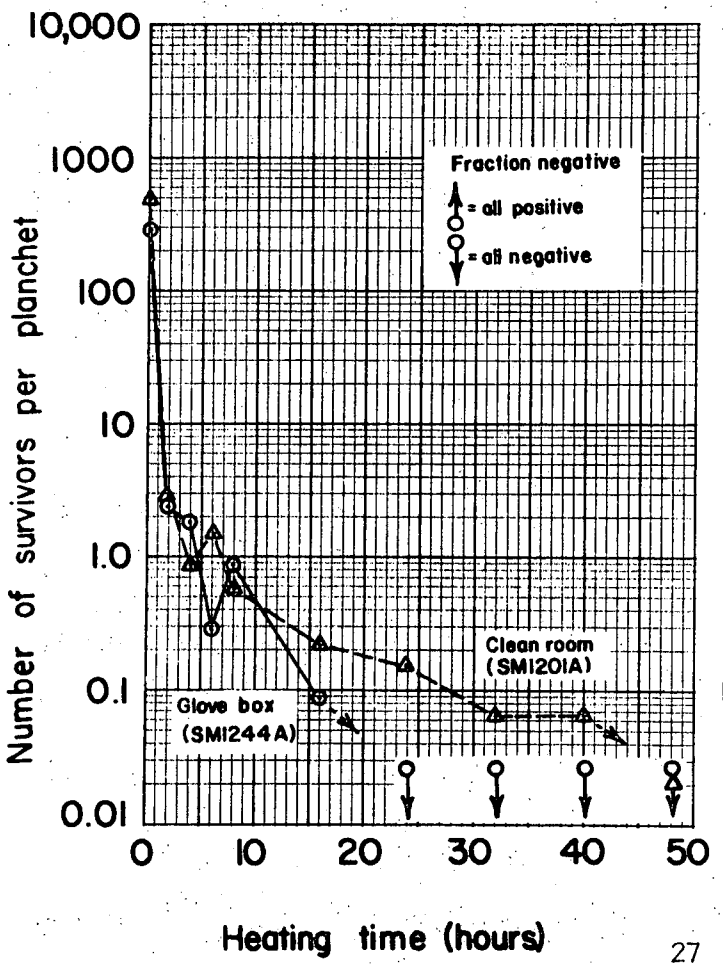

Figure 2.16 - Survival characteristics of organisms in soil heated in the clean room versus soil heated in the glove box

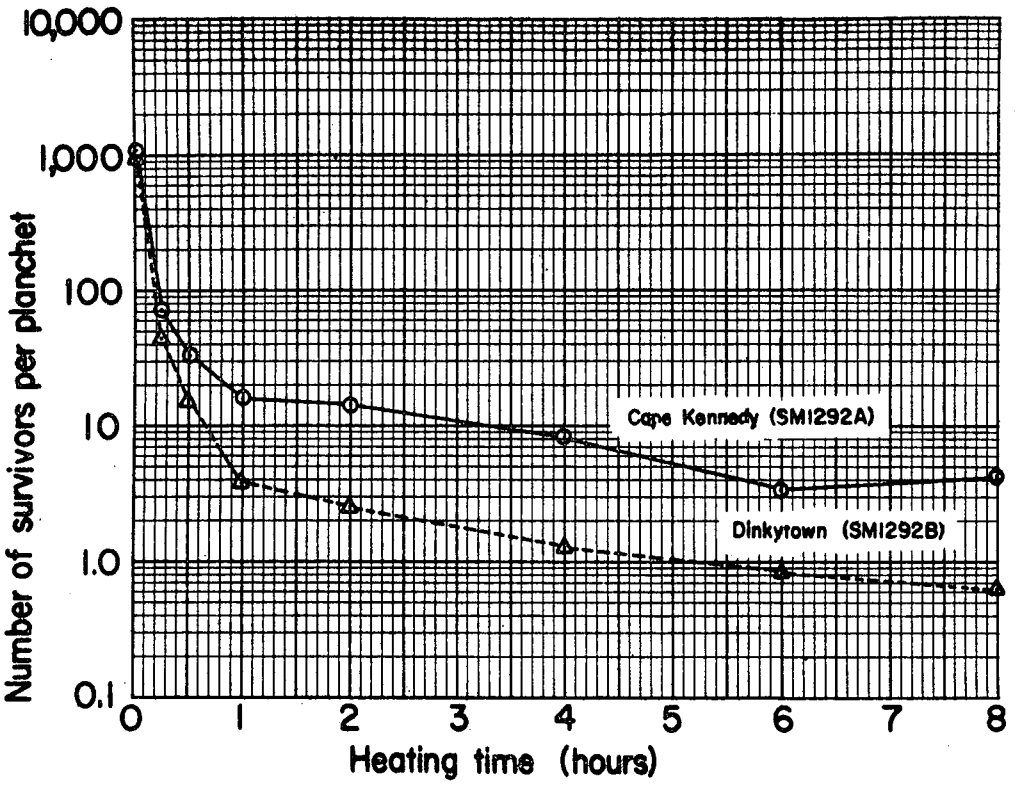

Figure 2.17 - Comparison of the survival of organisms from Cape Kennedy soil with survival in Dinkytown soil; survivor curve determined by plate count method

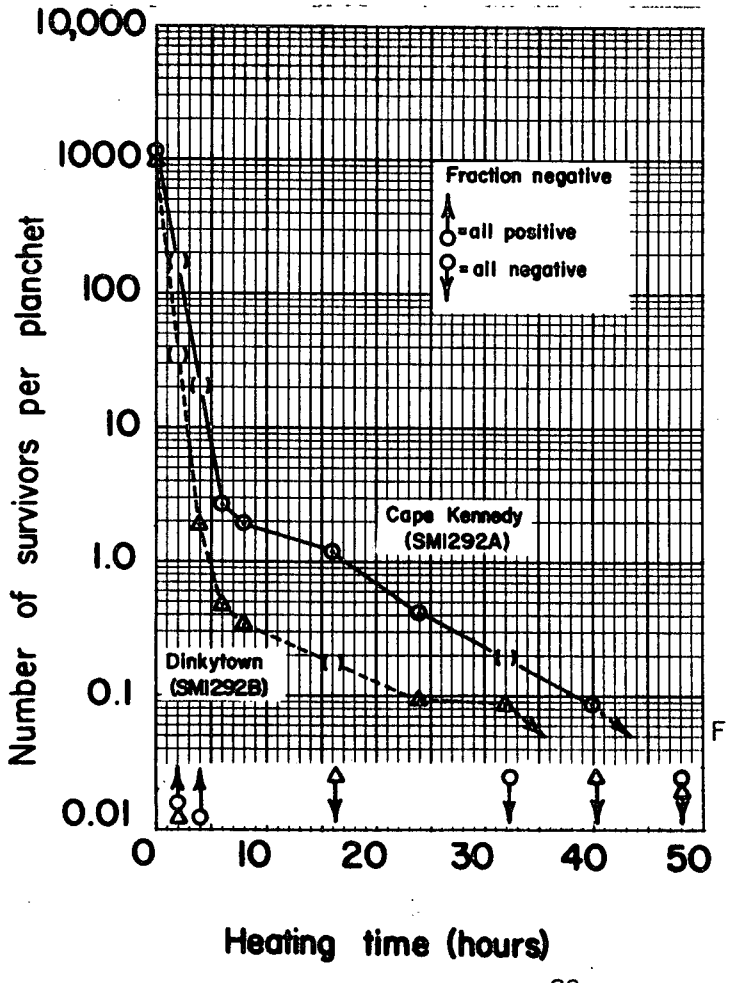

Figure 2.18 - Comparison of the survival of organisms in Cape Kennedy soil with survival in Dinkytown soil

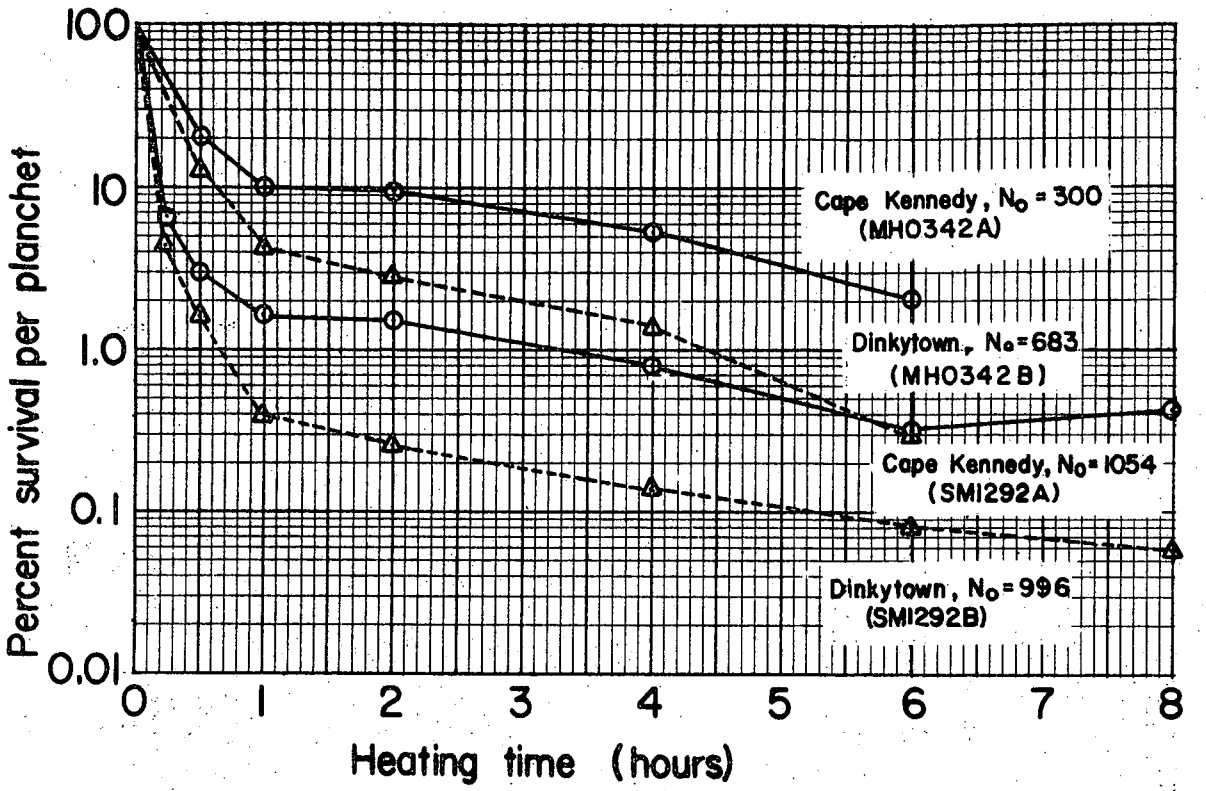

Figure 2.19 - Survivor curves of organisms in Cape Kennedy and Dinkytown soil, December, 1970, (MH0342) and October, 1971 (SM1292)

Discussion

It is doubtful that very many microorganisms exist in the soil as single units. It is more likely that microorganisms in the soil either have rock fragments, soil particles or crystals either adsorbed to them or they themselves are either adsorbed by or enclosed by these materials. The soil particles that we are dealing with in the majority of cases are made up of organic materials including spores, crystals, and small rock fragments, which are all cemented together. It is probable that in many cases this matrix is not broken down, even following our ultrasonic treatment. Therefore, the soil particle may remain essentially intact all the way to the final petri plate. In this case the soil particle actually will end up being one microbial colony-forming unit even though it may contain many microorganisms.

The number of microorganisms in a soil particle will undoubtedly be a function of particle size as well as the type of soil. We expect a larger number of microorganisms in a sample of large particles than in a sample of small particles where both were assayed using a plate count method. The net result then is that the actual number of microorganisms in a sample containing large soil particles may be considerably greater than the number indicated by a plate count and the actual number of microorganisms present in a similar sample made up of small soil particles.

It is also probable that the population of organisms associated with larger particles will be more resistant. The Phoenix CDC Laboratory has isolated a super-spore that is about 2.5 microns in diameter. This organism has an extremely high dry-heat resistance. It is possible that this super-spore with its 2.5 micron diameter would be normally attached to bits of soil. If we assume that it was attached to several bits of soil, the minimum size of the soil particle would be at least ten microns. As described above, a large particle will probably contain more microorganisms than a small particle and consequently, there is a greater chance that a super-spore will be in or associated with a large particle than with a small particle.

We are all very much aware of the role that water plays in the dry heat destruction of laboratory-cultured spores. We know that, starting with zero, as we increase the quantity of water in the spore during heating, the dry heat D-value increases. Soil particles contain organic material, crystals and small rock fragments and all of these elements either adsorb water or contain water of crystallization. In other words, there is a large amount of water present in soil particles. Some of this water will be driven off at dry heat sterilization temperatures. It is possible that this water that is driven off of the soil particles during heating will provide a protective atmosphere for the microorganisms associated with these soil particles. Since the amount of water that will be associated with the particles will increase with particle size, there will be more water and perhaps a greater protective effect associated with large particles than with small particles.

We believe that there are a number of very valid reasons why large soil particles show microbial survival; however, we are unable to pinpoint the precise reason for this phenomenon due to the lack of understanding of: 1) the exact role of water in protecting spores; 2) the exact rate of release of water from soil particles; and 3) the exact resistance characteristics of super-resistant spores.

As we put the several small pieces of dry heat destruction data together, a pattern begins to form. Extremely long survival times have been found for the microorganisms in garden soil, Cape Kennedy soil, or Dinkytown soil. At the other extreme, very short survival times have been found for the organisms that had fallen on strips in clean rooms or when clean spores were deposited on stainless steel surfaces. In this study, we showed that the size of the soil particle had an effect on microbial survival; organisms associated with large particles survived longer than those associated with smaller particles. In the overall picture we also see that: 1) there are some very dry-heat resistant organisms in soil; 2) the water condition of a spore during heating can alter the dry heat D-value of clean spores by a factor of 100; and 3) the relative humidity of the test environment may have little effect on organisms in a soil.

The first conclusion that can be drawn from all of the data is that organisms associated with particles of soil are many times more difficult tc kill than organisms on surfaces where soil is absent. The relative $F_{125°C}$-values for an 8-log reduction may vary by a factor of ten.

A second conclusion is that if we cannot keep out all soil we must try to keep out as much soil as possible and then have the remaining soil in the smallest particles possible. Hopefully, we will keep the particle size of the soil less than 1-3μ. It appears that the number of highly dry-heat resistant organisms in small soil particles is very low.

Summary

Analyses of our experimental results have led us to conclude that a longer dry heat process is required to sterilize microflora associated with larger particles than those associated with smaller particles. We believe that this phenomenon is due to any one or a combination of the following:

1. There is a larger number of microorganisms associated with each large particle than with each small particle.
2. There is a more dry-heat resistant population of microorganisms associated with large particles than with small particles.
3. Soil particles protect microorganisms against destruction by dry heat. The protective effect is a function of particle size and may be due to the water in the soil particles; the larger particles have a much greater protective effect than the small particles.

FUTURE WORK

In the future we plan to carry out a group of tests to determine the dry heat destruction characteristics of the soil microflora other than spores. We plan to carry out these studies at both 90°C and 125°C.

DRY HEAT DESTRUCTION RATES OF BACILLUS SUBTILIS VAR. NIGER IN A CLOSED SYSTEM

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INTRODUCTION

When we initiated our closed system experiments, they were all carried out at 125°C. During the past six months we have started to include lower temperatures in our studies. We have performed a number of experiments at high relative humidities at 90°C. There are two primary reasons for moving toward lower temperatures: 1) there was a possibility that NASA might consider the use of lower temperatures for sterilizing space hardware; and 2) we could possibly develop a better understanding of dry heat destruction characteristics in closed systems if we had temperature coefficient data available. This required that we have test results over a range of temperatures.

OBJECTIVES

In review, the objective of this project is to determine the dry heat D-values of microorganisms in closed systems. Specifically, it is to determine: 1) the relationship of spore D-value to spore water content; 2) the effect of atmospheric volume per spore on the D-value of spores with different initial water contents; 3) the effect of pressure on D-value, and 4) the effect of water adsorption and water vapor transfer characteristics of plastic materials on the D-values of spores encapsulated in plastic and spores on metal strips pressed against a plastic surface.

At this time we are reporting the results of studies in which Bacillus subtilis var. niger spores were heated at 90°C and relative humidities in the range of 30 to 100%. We are also reporting some preliminary results from experiments concerned with determining the effect of the length of conditioning time of the spores within the closed block system prior to heating on the subsequent dry heat destruction rate of these spores. We have evaluated the sealing efficiency of the closed block heating system. The overall problems of this system and the results of our studies are reported in Appendix D.

EXPERIMENTAL PROCEDURE

Using the stainless steel heat block system described in Progress Report #3,

a series of tests was carried out in which a measured quantity of liquid water was added to the cavity of the block just prior to sealing the block. The quantity of water added was calculated to produce specific relative humidities between 30 and 100% at the test temperature of 90°C.

In this series of experiments, the heat blocks used all had the 0.5-inch recess top (cavity depth=0.5", inside diameter=1.185", volume=.5544 in^3) and all had utilized a modified Teflon gasket (O.D.=1.625", I.D.=1.28", d=0.031") described in Appendix D. The eight cap-screws were torqued to 100-120 in-lbs.

Thoroughly cleaned and oven-sterilized (200°C for 2 hours) stainless steel discs were inoculated in the clean room (50% RH at 22°C) with 20 µl of a water suspension of spores using an Eppendorf push-button pipette. The concentration of the spore suspension was adjusted so that each deposit contained about 10^6 spores of Bacillus subtilis var. niger. Usually two separate deposits were made on a single disc; however, anywhere from one to four deposits can be made. The cleaned and heat-dried (125°C for 2 hours) blocks, cap screws, and gaskets along with the inoculated discs and sterile, stainless-steel, spacer discs were placed in the clean room to equilibrate overnight (18 hours) prior to assembly and testing.

The blocks were assembled according to the following assembly schedule: A Teflon gasket was inserted into the holding groove in each of the block top units. A sterile spacer disc was placed in the well of each bottom unit to facilitate later removal of the inoculated discs. An inoculated disc was placed directly on top of the sterile spacer disc. An amount of distilled water calculated to give a specific RH at 90°C was deposited by means of a micro-syringe on the inoculated disc equidistant between the two spore deposits. The top unit of the block was then placed on the bottom unit and the two pieces were bolted together using Allen-head cap screws. The cap-screws were first tightened by hand using an Allen wrench. The block-sealing screws were then torqued to about 120 in-lbs. as the final step in the assembly. The assembly steps following deposition of the water drop were performed as quickly as possible so that water loss by evaporation from the block cavity into the clean room was minimized.

Heating was carried out in an agitated water bath maintained at 90°C. At the end of the heating period, the block units were cooled by immersing them in an ice water bath for 1.5 minutes.

After the block unit had been heated and cooled, it was carefully disassembled and the inoculated disc was removed. The spore deposits were separated by aseptically cutting the disc. Each portion of the disc containing one spore deposit was then assayed for survivors according to NASA standard procedures.

The following tests were all carried out at 90°C:

1. Tests to obtain survivor curves at relative humidities of 30, 45, 60, 75, and 90%.
2. Tests to determine the relative number of surviving organisms after a constant heat treatment of 20 minutes as a function of the quantity of water added to the block. Water conditions evaluated were those which produce relative humidities of 80, 85, 90, 95, 100, 105, 110, 120, 140 and 200%. We are using 105%, 110, etc., to indicate the quantity of water added relative to the amount of water needed to produce a saturated atmosphere in the block cavity.
3. Tests to develop survivor curves which show whether there was a difference with respect to shape, slope and intercept ratio of survivor curves for spores located in cups with liquid water present and for spores which were located in cups that had been subjected to a relative humidity of 100%.
4. Tests using a constant heating time of 80 minutes to determine the effect of the elapsed time (conditioning time) between sealing the block and placing the block into the water bath on the number of surviving organisms. This conditioning time varied from 4 to 1425 minutes.

RESULTS AND DISCUSSION

Test 1

The results of the survivor curve experiments at 90°C at 30, 45, 60, 75 and 90% RH are shown in Figure 3.1. Needless to say, increasing the relative humidity decreased the survival time or in terms of D-values, increasing the relative humidity resulted in a smaller D-value. It is interesting to note in Figure 3.1 that the slope of the survivor curve increases by approximately a factor of three for each additional 15% RH increment from 45 to 90%.

Test 2

In Figure 3.2 we show the number of organisms surviving after a 20-minute heating time at 90°C. The quantities of water present in the heat blocks were all in the neighborhood of that necessary to produce a saturated condition (100% RH). We do not believe that the numbers of survivors are significantly different among the several tests. In general, the number of survivors (about 2×10^3) seems to remain relatively constant for 100, 105, 110, 120 and 140% of the quantity of water necessary to produce saturation. Fewer survivors were detected for the 85, 90 and 200% RH conditions, although it is questionable whether these were significantly different from the others.

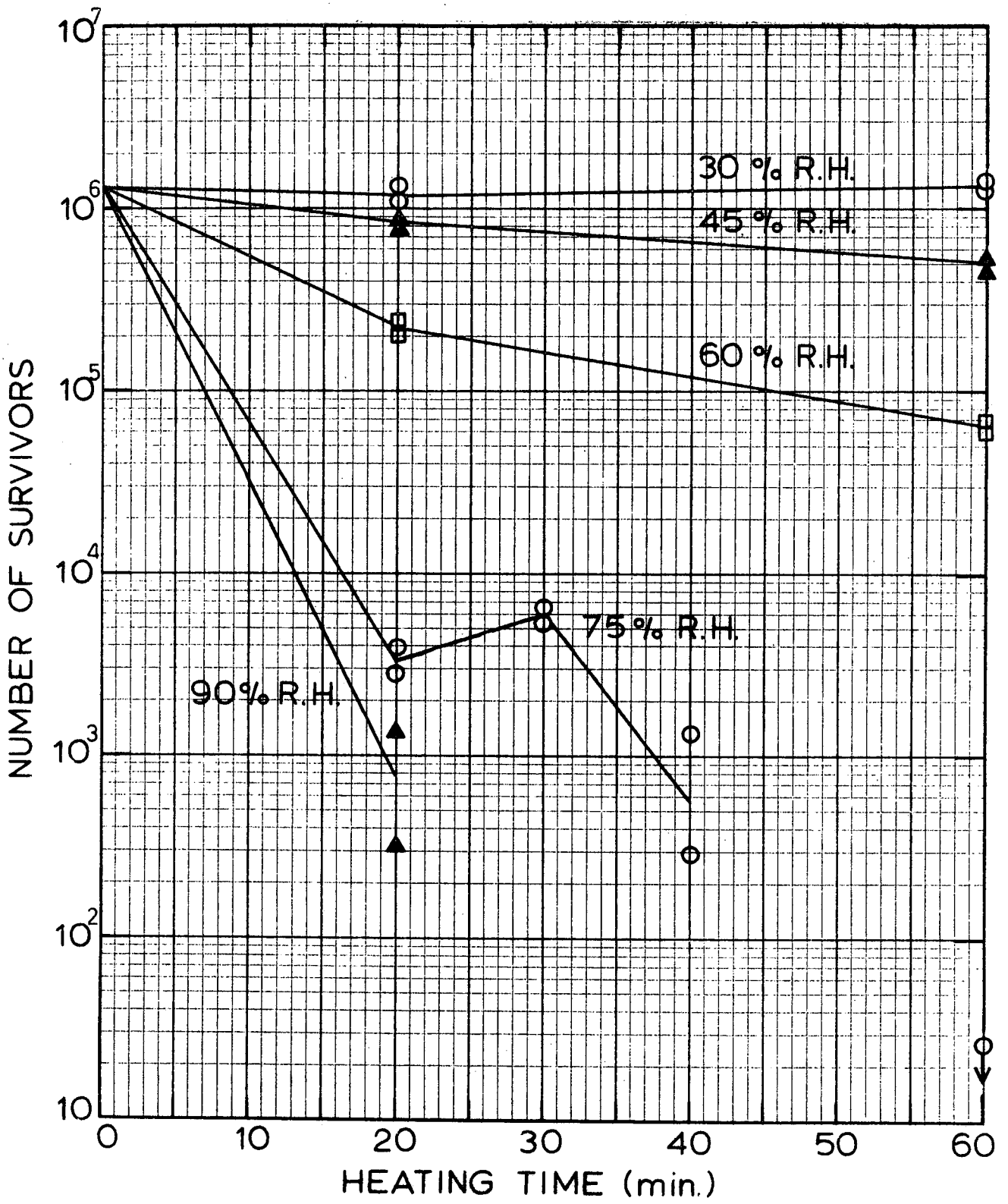

Figure 3.1 - Survivor curves at 90°C for relative humidities of 30, 45, 60, 75, and 90%

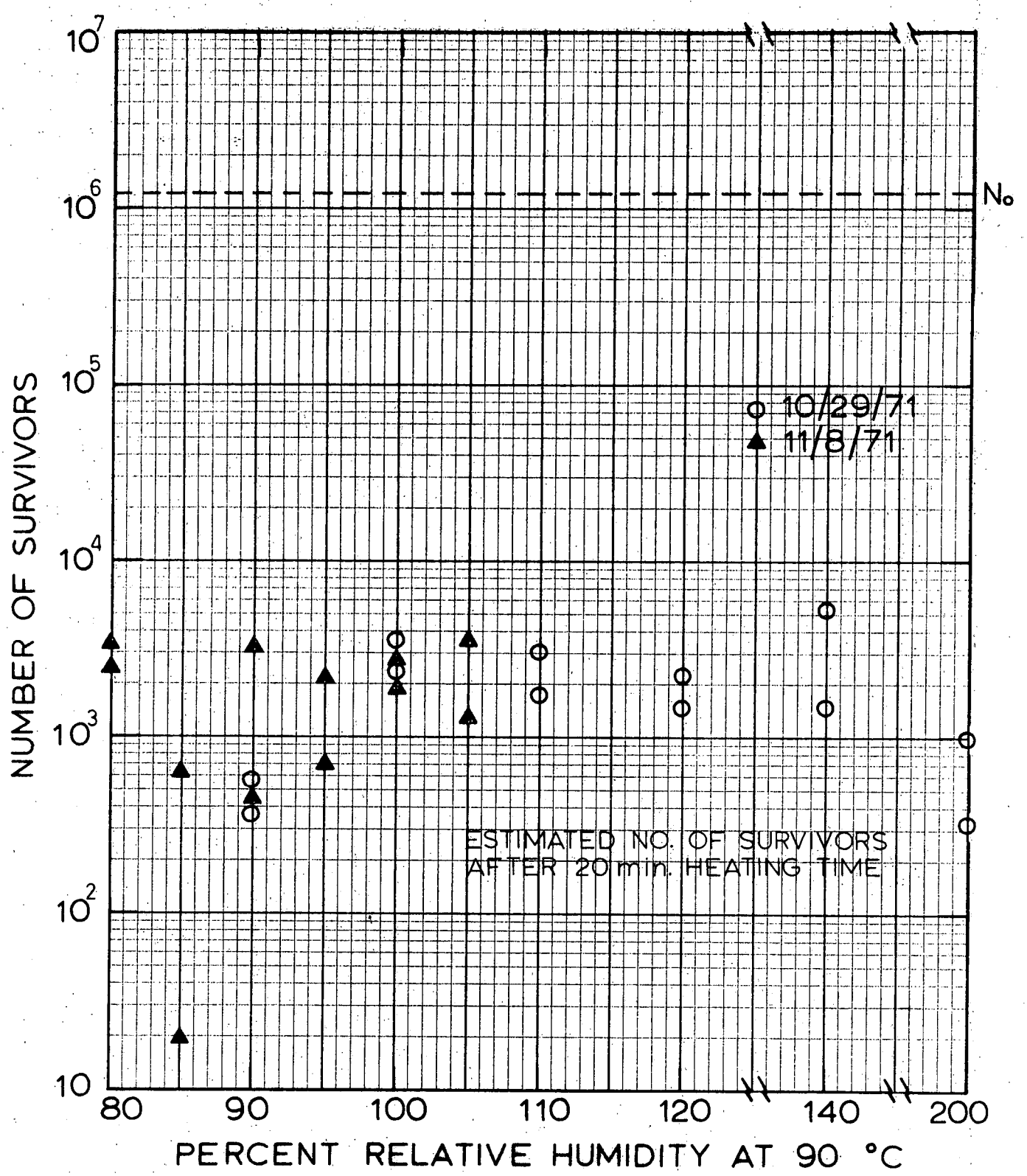

Figure 3.2 - Survivor data for tests at a series of water conditions. The data at 105, 120, 140 and 200 represent a water condition 1.05, 1.20, 1.40 and 2.00 times the quantity of water at 100% relative humidity.

Test 3

The results of the survivor curve experiments in Test 3 were most surprising. As shown in Figure 3.3, this test compared survival of spores in liquid water and spores in a saturated atmosphere. It is rather obvious from this data that when the spores are in water the results are totally different from those that are obtained when the spores are in a 100% RH atmosphere. Not only are the results different in magnitude but the variability in the results for the two tests are quite different. Variability is small for the tests where the spores are in water; whereas, there is considerably more variation for the 100% RH atmosphere.

These data suggest that when spores are in water, they are under quite different physiological conditions than when they are in a 100% RH atmosphere. It would be interesting to find out if equilibration time (see Test 4) has an effect on the results when the spores are dry and then subjected to 100% RH. We might assume theoretically that for some equilibration time the results should be the same; however, then we have to deal with the possible historesus that may exist relative to the location of certain physiological components of the cell when the cell is in water versus when it is dry and subjected to 100% humidity and is in equilibrium with this atmosphere.

Test 4

The results of the series of tests carried out to determine the effect of the conditioning time on the number of survivors are shown in Figures 3.4, 3.5, and 3.6. In Figure 3.4 we show the profile of the number of survivors after a constant 80-minute heat treatment at 90°C, 75% RH as a function of the conditioning time in the block. In Figure 3.5 we have expanded the time scale to show the results for short conditioning periods. In Figure 3.6 we have used a linearized survivor curve plot to show how three conditioning times, 4, 44 and 1425 minutes, would produce grossly different projected D-values.

We believe that it is worthwhile to state why we decided to examine conditioning time in the heat blocks. In carrying out a series of tests, variable results were obtained. Proceeding on the basis that we must believe what the spores are telling us, we carefully reviewed the experimental procedure. From an analysis of our results in terms of the time of assembly, we deduced that what first appeared to be a rather random variation in our results was actually a change in the magnitude of the results as a function of the estimated time from sealing to heating the blocks. Immediately after observing this phenomenon, we proceeded to enter in the data log book the time at which the block was sealed and the time at which the block was placed in the water bath. After confirming that conditioning time played a role in

spore survival we then proceeded to design Test 4.

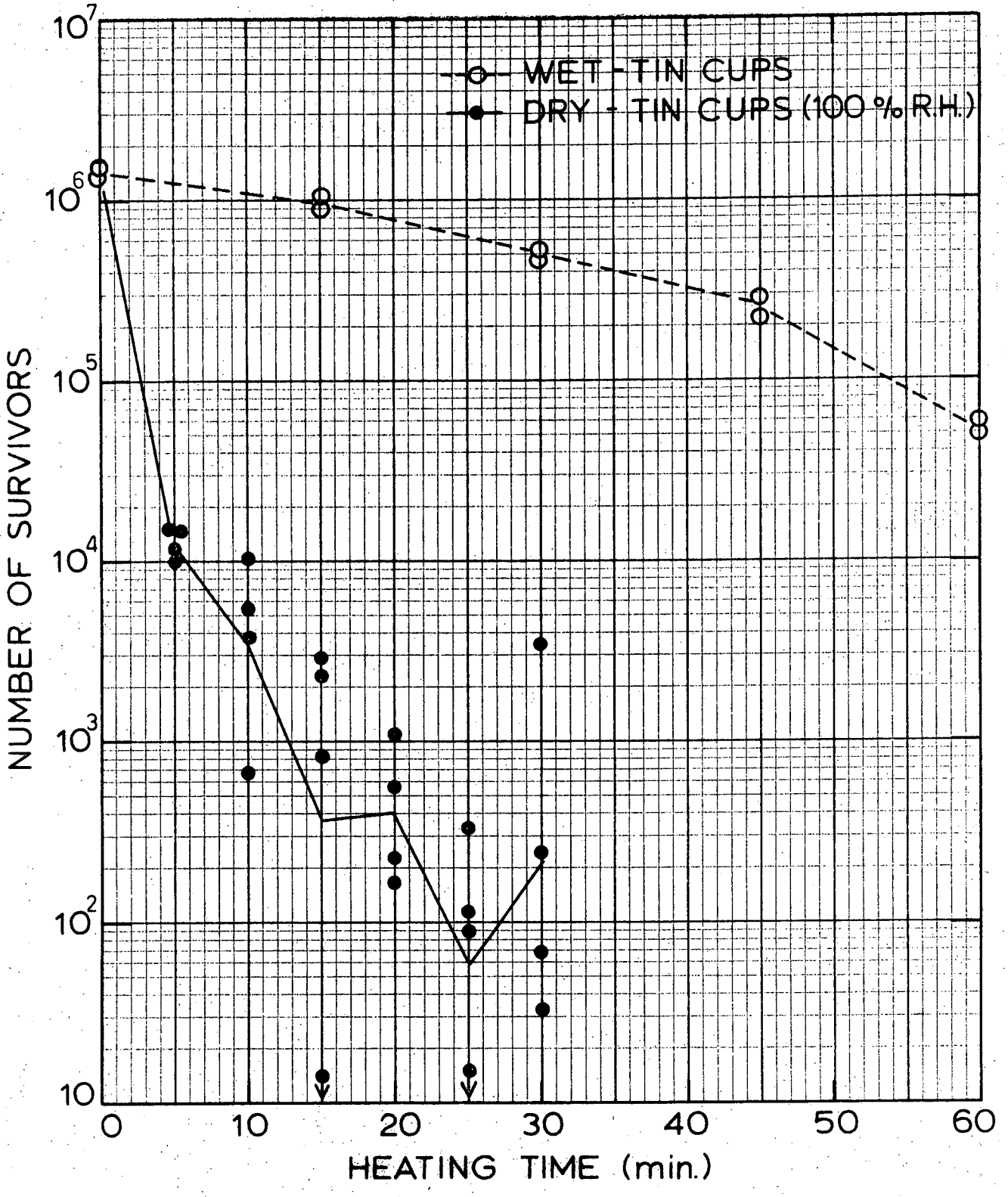

Figure 3.3 - Survivor data at 90°C for spores in water (wet) and in a 100% relative humidity environment (dry)

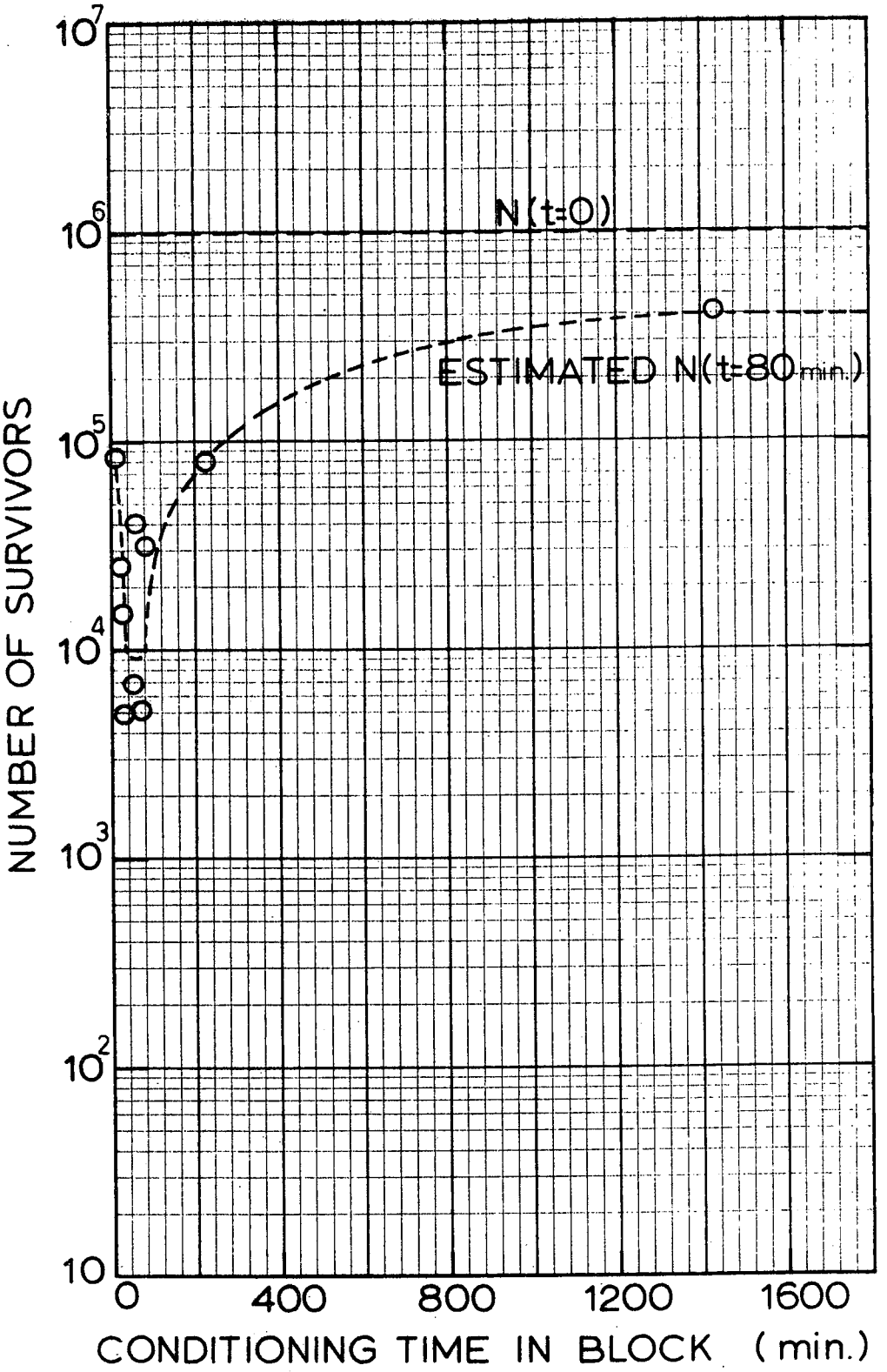

Figure 3.4 - Survivor data for spores heated for 80 minutes at 90°C in the heat block with water added to produce 75% relative humidity as a function of the conditioning time in the block

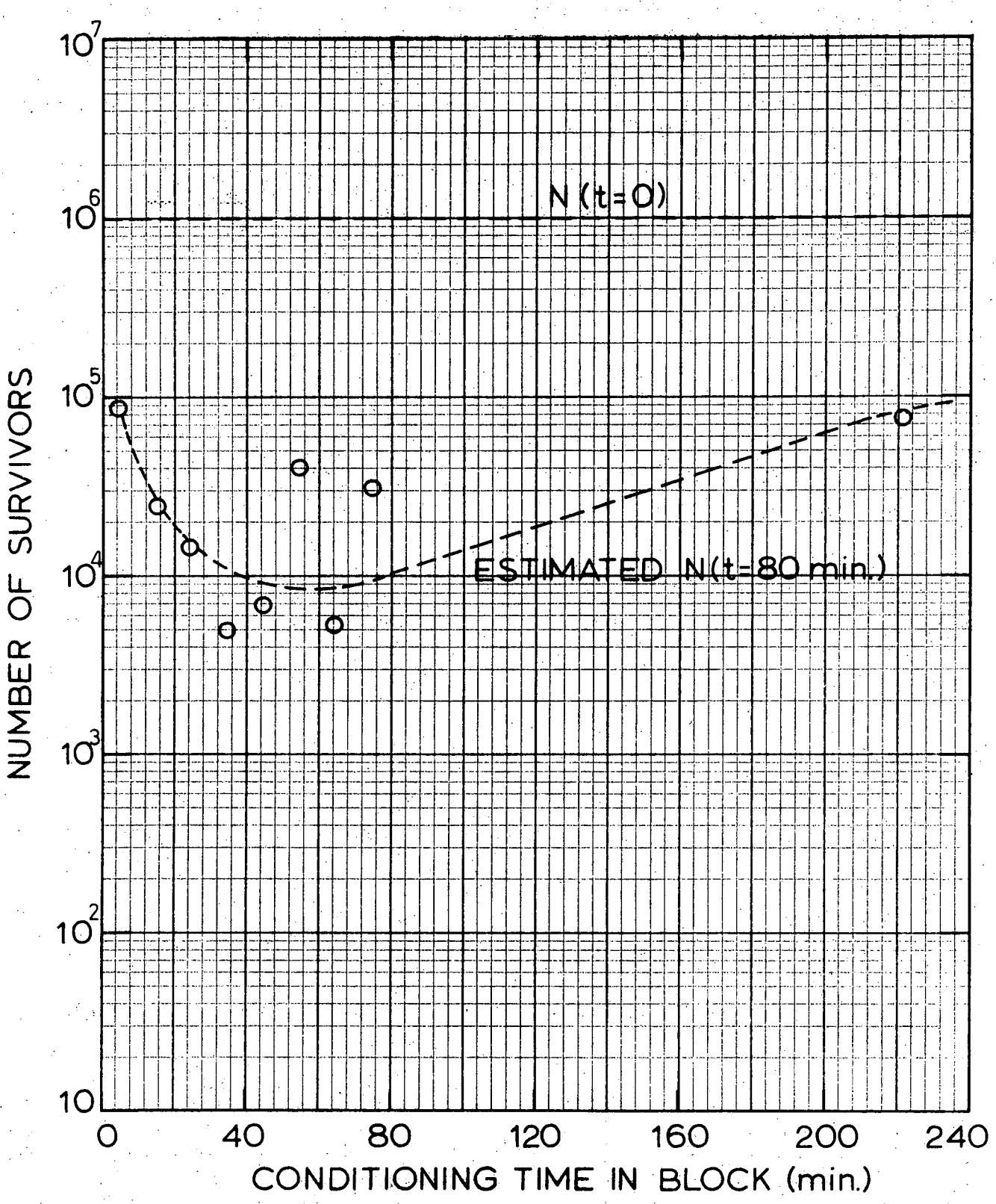

Figure 3.5 - Survivor data for spores heated for 80 minutes at 90°C in the heat block with water added to produce 75% relative humidity as a function of the conditioning time in the block. Scale expanded to show only the data up to 222 minutes.

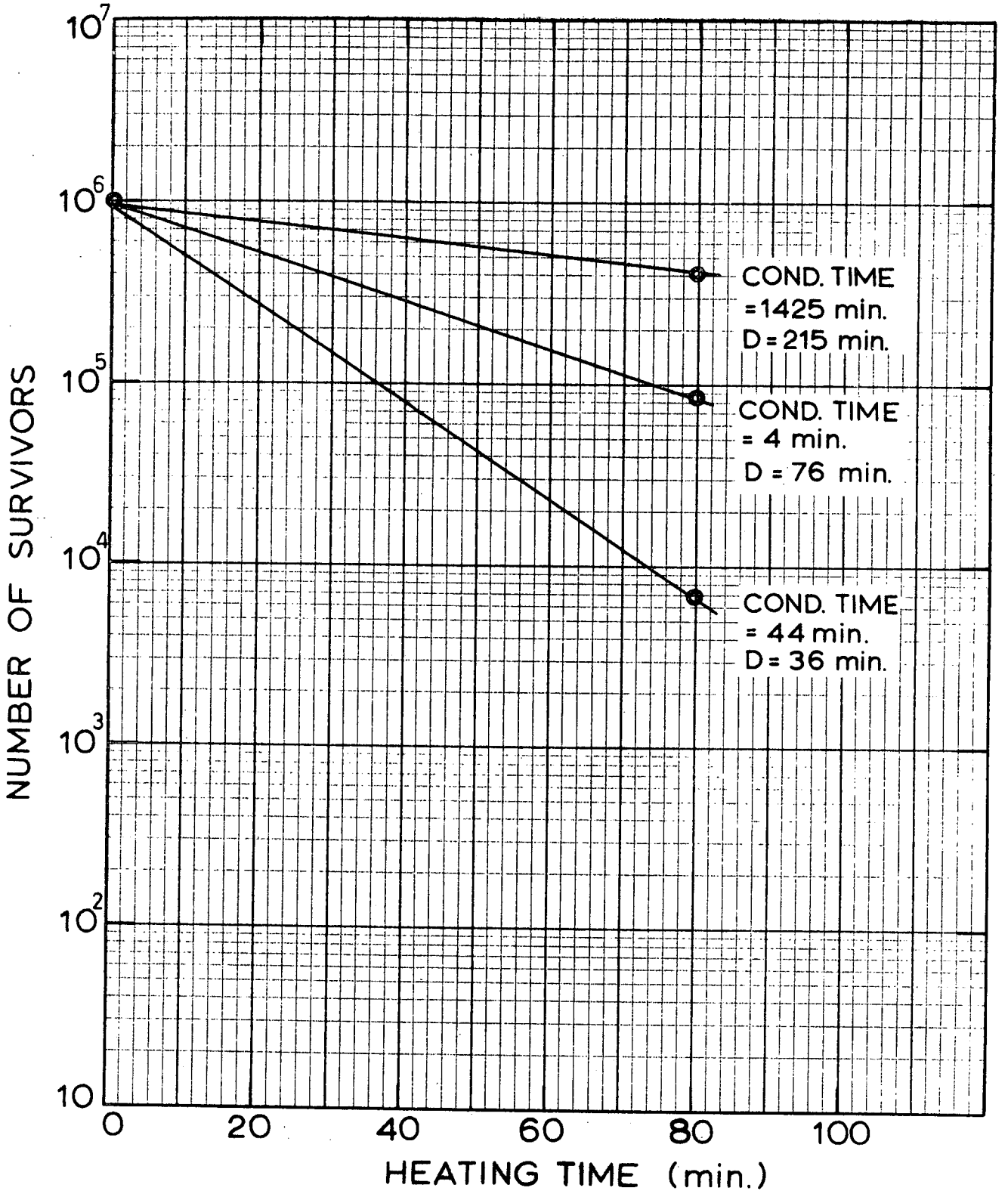

Figure 3.6 - Two-point survivor curves for spores heated at 90°C with 75% relative humidity in the heat block for conditioning times of 4, 44 and 1425 minutes.

At this time we have not developed any theory for explaining this influence of conditioning time in the block. We hope to alert the reader to the fact that, in this system where we are attempting to evaluate a relative humidity of 75% at 90°C, a liquid water drop is added to the block at ambient conditions; thus, the quantity of water added is in all cases more than enough to produce 100% RH at the assembly temperature. Since the spores were previously equilibrated in an atmosphere of 22°C and 50% RH, we would assume that when they are in contact with an atmosphere near 100% RH at 22°C, they will absorb water. The rate of water absorption will depend on the vapor pressure difference between the water in the spore and in the atmosphere. A second variable is that the vapor pressure in the atmosphere surrounding the spore will be a function of the mass transfer rate between the deposited water droplet and the atmosphere as well as the absorption rate of the spores. Since heat is required to evaporate water, small changes in temperature will have a major effect on this evaporation rate. Without going further, we believe that we can make a good case for the fact that we are dealing with a rather involved mechanism in which any of several parameters could be limiting. Needless to say, this entire area will require further investigation.

General discussion

The results of these four tests suggest that at relative humidities above 60% at 90°C there are undoubtedly variables involved in the overall spore destruction pattern that have not heretofore been identified. The lethality of stress conditions may involve the sequence of events which would affect the interaction between water and the spore either immediately prior to heating or at the onset of heating. Perhaps there is a clue to this effect by observing that the D-value is small when the water activity is near zero and reaches a maximum at some intermediate water activity and decreases again to a small value as we approach a water activity of one. Another fact that warrants consideration is that with dry spores there is an immediate one- or two-log drop in the viable count prior to a decreased rate of destruction; whereas, with wet spores we tend to have a low initial destruction rate followed by a higher destruction rate at longer times.

As a final point, we might observe that perhaps a sequence of wetting and drying conditions may make the spores more susceptible to destruction by heat than a constant temperature in either a wet or dry environment.

SUMMARY

We have found that at 90°C and relative humidities of 30 to 100%, the rate of destruction of *Bacillus subtilis* var. *niger* spores increases as the relative humidity

increases, which is consistent with established theory.

At relative humidities of from 70 to 100% the length of the time interval between the sealing of the spores inside the heat block and the time when the heat block is placed in the 90°C water bath has an effect on the number of spores that survive a given heating time.

FUTURE WORK

During the next six months, studies will be continued examining the effects of low treatment temperatures at high relative humidities. Specifically, we will study treatment temperatures of 75, 90 and 105°C and relative humidities from 55 to 100% including liquid water. Work will begin with treatment temperatures of 90 and 75°C.

Efforts will be made to establish a clearer relationship between the effects of relative humidity and the effects of the time between sealing the heat block and the beginning of the heating period.

EFFECT OF COMBINED HEAT AND RADIATION ON MICROBIAL DESTRUCTION

Project Personnel: D. Fisher, D. Barber, I. Pflug
Division of Environmental Health

INTRODUCTION

Sandia Laboratories of Albuquerque, New Mexico, have been investigating the effect of dry heat and gamma radiation on the survival rate of a uniform crop of Bacillus subtilis var. niger spores. They have reported a synergistic effect when the spores were subjected simultaneously to both dry heat and low levels of gamma radiation. The NASA Planetary Quarantine Office has asked this laboratory also to investigate the sterilization attributes of combined dry heat and gamma radiation.

This project was initiated in June of 1971 and this report describes our activities to date. During the past six months we have designed and constructed a controlled environmental system which is now being peformance tested. We have also mapped the radiation field of the University of Minnesota Cesium-137 radiation source that will be used in this study.

OBJECTIVES

The objectives of this project are as follows: 1) to investigate the survival of spores on surfaces at various temperatures in a precisely controlled environmental system, 2) to determine the rate of destruction of these spores at ambient temperature when subjected to gamma radiation and 3) to determine the rate of destruction of these spores when they are subjected to combined gamma radiation and thermal stress.

DISCUSSION

Environmental System

A precisely controlled environmental system has been designed and built and at the present time is undergoing final testing. This system is shown schematically in Figure 4.1. Constant temperature and humidity conditions are maintained by controlling the saturation temperature and the dry bulb temperature of the circulating airstream.

The path of the airstream through the system and the treatment applied to the air are as follows: The air passes through a spray chamber where it is saturated. The amount of water evaporated is far in excess of the amount sprayed so evaporative cooling is negligible. The residence time of the air in the spray chamber is

sufficiently long so that the air temperature is the same as the spray temperature. The water temperature in the spray reservoir is maintained using heaters controlled by a RFL proportional controller. A blower draws the air from the spray chamber past an air heater and blows the air past the spores, over wet and dry bulb thermocouples, and through a calibrated orifice. The air heater raises the temperature of the airstream sufficiently above the saturation temperature so that condensation will not occur between the spray chamber and the environmental chamber containing the bacteria spores. Upon entering the environmental chamber, the air passes first through a heating section where it is heated to the temperature of the environmental chamber. The bacteria spores are deposited on stainless steel planchets which are mounted in the environmental chamber normal to the airstream. The electrical heaters in the chamber are controlled by another RFL proportional controller. When the airstream leaves the environmental chamber, it passes directly into a chamber containing the thermocouples which monitor the wet and dry bulb temperature of the airstream. The airstream then passes through a calibrated orifice where the volumetric flow rate is measured. The flow rate is adjusted by changing the speed of the fan.

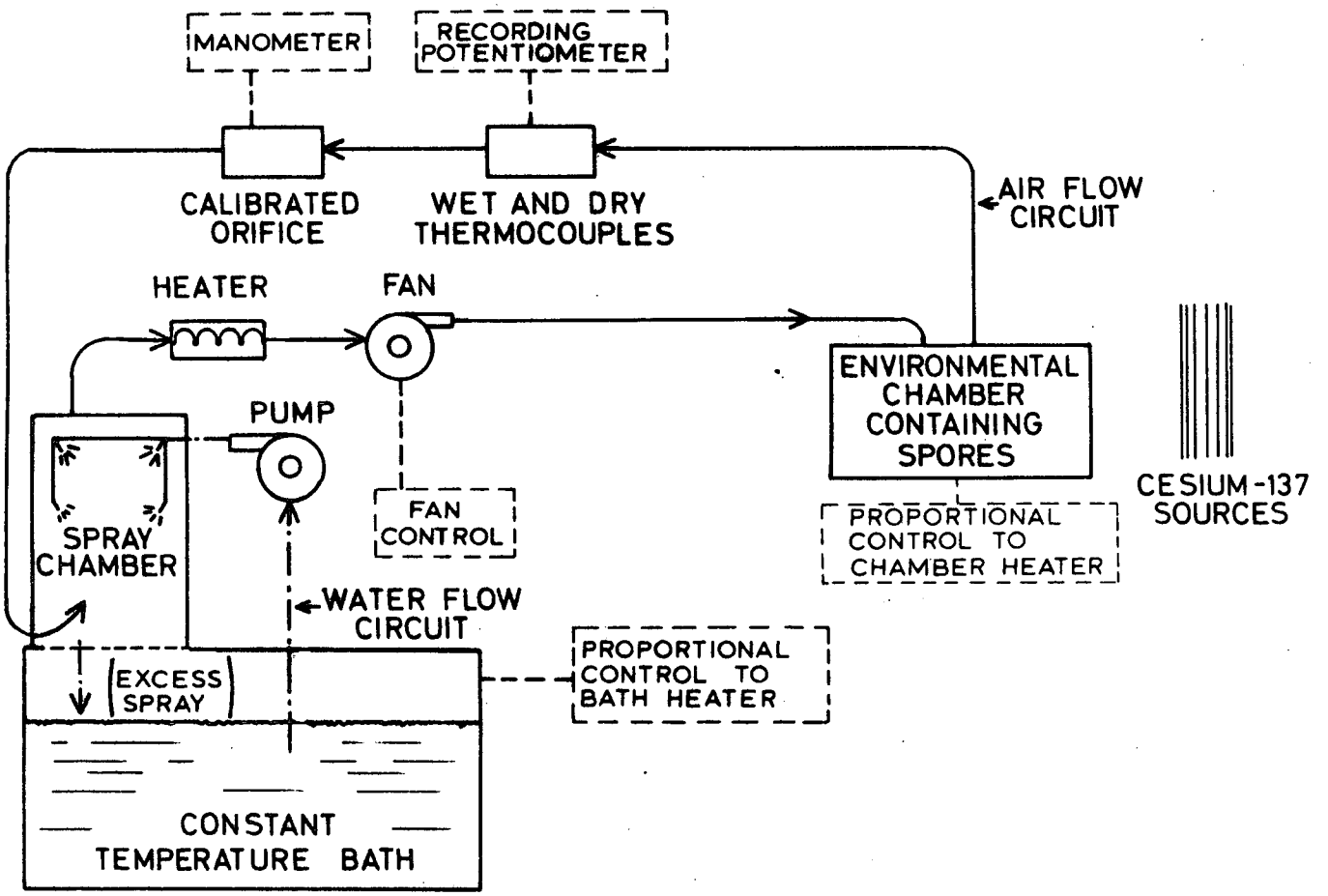

Figure 4.1 - Schematic flow chart for the environmental system that will be used in thermoradiation experiments.

The system has been designed so that the environmental chamber can be easily separated from the remainder of the system in order that the loading and unloading of the planchets can be carried out in a clean microbiological area.

A special start-up procedure is required if condensation on the planchets is to be avoided. This special procedure is necessary in our system since the dew point of the circulating airstream is above the ambient temperature. We plan to use the following start-up procedure: with the environmental chamber by-passed, the air circulating system will be heated to a stable temperature greater than the dew point, using the auxiliary heater. When the temperature has stabilized, the heaters in the environmental chamber will be activated to rapidly bring the chamber's temperature to the desired test level. When the environmental chamber reaches test temperature it will be connected to the airflow system and the spray will be turned on. Using this procedure we hope to avoid the problem of condensation in the system during the initial part of the experiment while rapidly achieving test psychrometric conditions.

A thermoluminescent dosimetry (TLD) system using $Li_2B_4O_7$:Mn powder has been calibrated to measure the radiation dose applied to bacteria spores. Capsules which are impervious to light and water contain the TLD powder and are located in the same isodose plane as the spore samples.

The design parameters of the system are:

1. dry bulb temperature - controlled to within 0.1°C using the proportional heater on the airstream. Spacial variation of temperatures within the environmental chamber is ±.2°C. Temperatures from 22°C to 125°C may be maintained in this system.
2. relative humidity, wet bulb temperature or saturation temperature - controlled by the proportional controller used for maintaining the temperature of the water in the spray reservoir and chamber. The saturation temperature may be controlled to within 0.1°C and can range from 16°C to 100°C. The controllable psychrometric conditions are shown in Figure 4.2.
3. airflow rate - monitored by the pressure drop across the calibrated orifice and regulated with the blower-control. The bulk flow rate can vary from 0 to 3.3 ft^3/min such that the linear velocity in the environmental chamber ranges from 0 to 32 ft/min.
4. gamma radiation dose rate - adjusted by changing the position of the spore sample relative to the Cesium source.

Spores

Bacillus subtilis var. niger spore crop AAHF will be used in this study.

The stainless steel planchets will be assayed using NASA standard procedures. The results will be reported in the form of survivor curves.

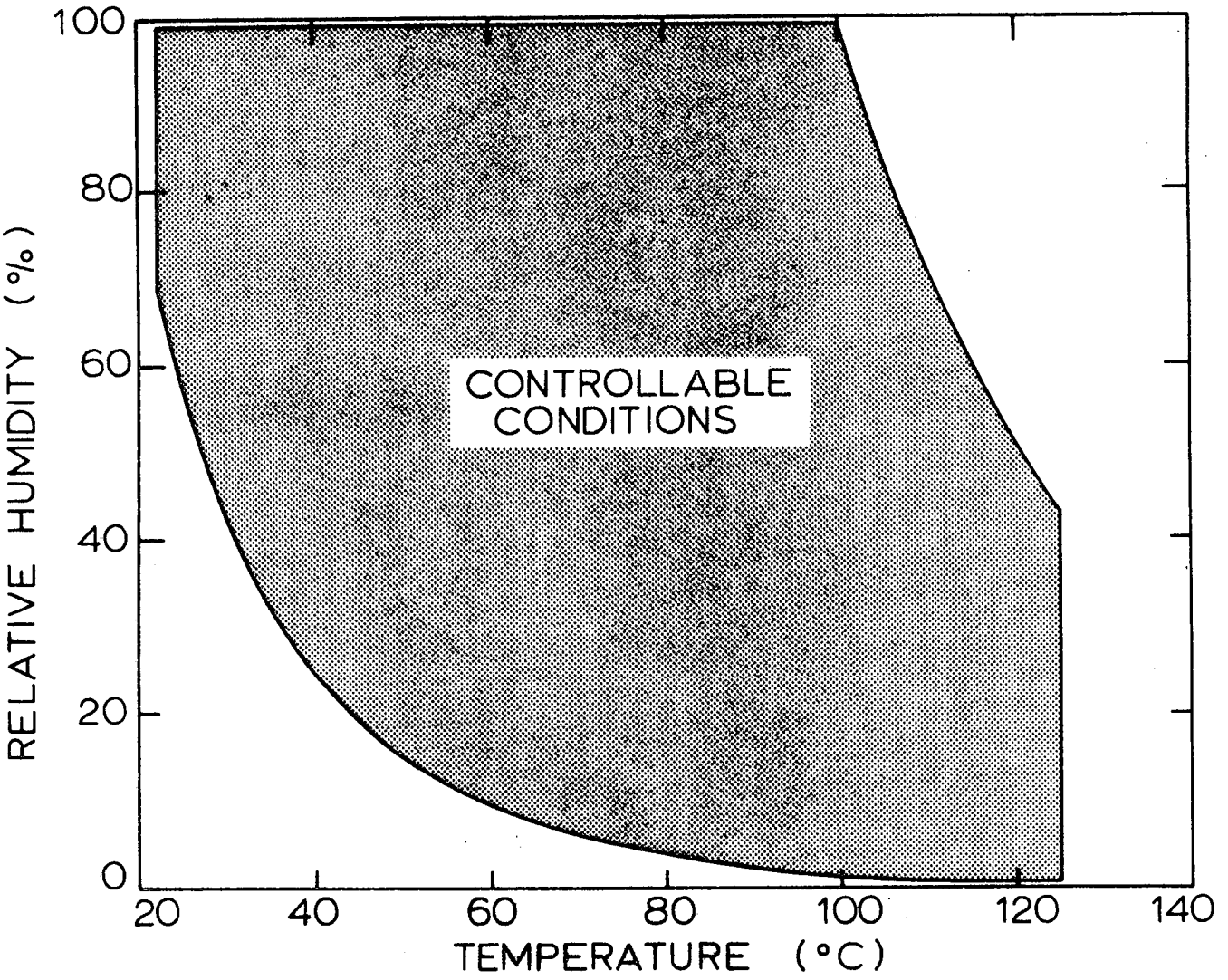

Figure 4.2 - Psychrometric conditions which can be maintained in the environmental system

Radiation Field

The Gamma Irradiation Facility at the University of Minnesota will be used in this study. The radiation field external to a cylindrical array of Cesium-137 line sources has been mapped both theoretically and experimentally. This region had not been adequately mapped since most earlier work performed in the facility required higher dose levels achievable only inside the cylindrical array -- a region where

the radiation levels are well-known.

The radiation levels were calculated using well-established computation schemes.[1] Experimental results obtained with chemical dosimetry (Fricke) and measurements using a calibrated Victoreen rate meter agreed with the theoretical values.

FUTURE WORK

After a final testing of the environmental unit, we will begin the bacteriological testing portion of this project. We plan to:

1) generate survivor curves when the spores are subjected to thermal stress only. The proposed temperature levels we will use are 60, 75 and 90°C.
2) examine the lethal effect of radiation on spores at ambient temperatures. We intend to use radiation levels of 5, 10, and 20 kR/hr.
3) determine the survivor curves of spores when subjected to various combinations of the above-mentioned temperatures and gamma radiation levels.

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[1] Engineering Compendium on Radiation Shielding. Vol. I, Springer-Verlag, 1968.

APPENDIX A: DESIGN AND PROCEDURE FOR SOIL EXPERIMENTS

The tests to determine the relative survival of spores associated with soil as a function of the size of the soil particles were carried out according to the following design and procedure.

An experimental design was developed which employed a number of soil suspensions, heating conditions, heating times, boats and planchets. The number of each of these items was determined following consideration of the objective of the experiment, expected sources of variation and available resources.

The objective of each experiment was to compare the survival characteristics of spores in different soil suspensions. The design emphasized the comparison within each heating time.

Expected sources of variation were the physical characteristics of the boats, the position of the boats on the hot plate, the order of deposition and the order of treatment. The test plan included a randomization scheme designed to reduce the chance of systematic effects due to these sources.

The available resources included a supply of copper boats. The copper boats used in these experiments were manufactured at three different times from different pieces of copper bar stock. Since the boat unit was critical in our heat flow pattern, steps were taken to minimize any effect due to differences between boat lots. Boats from a single lot were used for a single replicate of cell suspensions, when three replicates were used boats from three lots were used as shown in Figure 1.

Preparation of a test plan

Two randomization schemes for boat assignment were prepared for each experiment; one scheme was prepared for that part of the experiment using the plate count assay procedure and the second scheme was prepared for the fraction negative assay method.

Each randomization scheme was carried out in the same way. An example of a typical randomization scheme where the survival characteristics of spores in three different soil suspensions (A,B,C) were compared was as follows: The first step was to randomize boats of a single lot to heating times and suspensions using a table of random numbers. When the boats within all three lots had been randomly assigned to treatment, the completed randomization scheme was similar to that shown in Figure 1.

Experiment: SMI187 Assay method: plates

Heating time (minutes)	Boat lot #	Boat numbers for suspensions		
		A	B	C
0	1	57	20	56
	2	66	97	74
	3	147	110	119
15	1	55	29	26
	2	61	107	109
	3	143	152	148
30	1	54	37	30
⋮	⋮	⋮	⋮	⋮

Figure 1 - Randomization scheme for boat assignment

Schedule for deposition

Following assignment of the boats to heating time and suspension, the order of deposition was determined. All boats for one suspension were listed on a schedule for deposition similar to Figure 2. This figure represents a tray containing the boats. The boats were separated into groups according to the method of microbial assay. Boats were arranged on the tray by boat number within the assay method as shown in Figure 2. Thus all boats to be processed for plate counts appear in numerical order beginning at the upper left-hand corner of the sheet and continuing in a snake-like fashion. The boats assigned to the fraction negative assay method appear after those assigned to plate counts.

Experiment: SMI187 Suspension: A

21 P	25 P	36 P	50 P	...	104 P	121 P	128 P	132 P
83 FN	82 FN	81 FN	69 FN	...	149 P	147 P	143 P	137 P
86 FN	103 FN	105 FN	106 FN					

P = code for plate count assay method

FN = code for fraction negative method

Figure 2 - Deposition schedule

Schedule for heating

A schedule for heating similar to that shown in Figure 3 was also prepared. Boats were grouped according to treatment and lot. All boats assigned to the same heating time and originating from the same lot were grouped together. This meant that each suspension was represented in each group. The estimated time of day that each group of boats was to be placed on the hot plate was included. (The placement time was approximately the same as the estimated time.) The removal time was calculated from the actual placement time and recorded. Placement time was not randomized.

Experiment: SMI187 Assay method: plates

20	56	57	66		54	75	77	98
	0-			...			30 min	8:00A
								8:45A
121	141	150	6		149	3	21	45
	30 min	8:15A		...			240 min	9:45A
		8:46A						1:46P
64	95	102	113		104	128	151	158
	240 min	9:15A		...			480 min	8:00A
								3:52P

Figure 3 - Boat heating schedule

Carrying out the experiment

The boats were arranged on separate trays according to the schedule for deposition. The following procedure was used:

1. Planchets were placed on boats.
2. An Eppendorf microliter pipette was used for depositing 0.01 ml of preparation on each planchet. The order of deposition made a snake-like pattern. The deposits began with the first row of planchets on the first row of boats and proceeded from left to right. Next the first row of planchets on the second row of boats was inoculated from right to left. After one planchet had been inoculated on each boat the process was repeated for the second row of planchets and continued until deposits had been made on all planchets.
3. The boats containing inoculated planchets were then transferred to trays and placed in the order given in the randomization scheme for heat treatment.

4. The deposited spores were conditioned in the laminar downflow clean room and were shielded from the direct airflow. Spores used in the plate count assay method were conditioned 16 to 24 hours while those used in the fraction negative assay were conditioned 16 to 72 hours.
5. At treatment time, a minimum interval of one minute was maintained between placing groups of boats on the hot plate. All groups associated with one heating period were placed on the hot plate according to the arrangement given in the randomization schedule for heat treatment. The arrangement of boats the hot plate is shown in Figure 4.

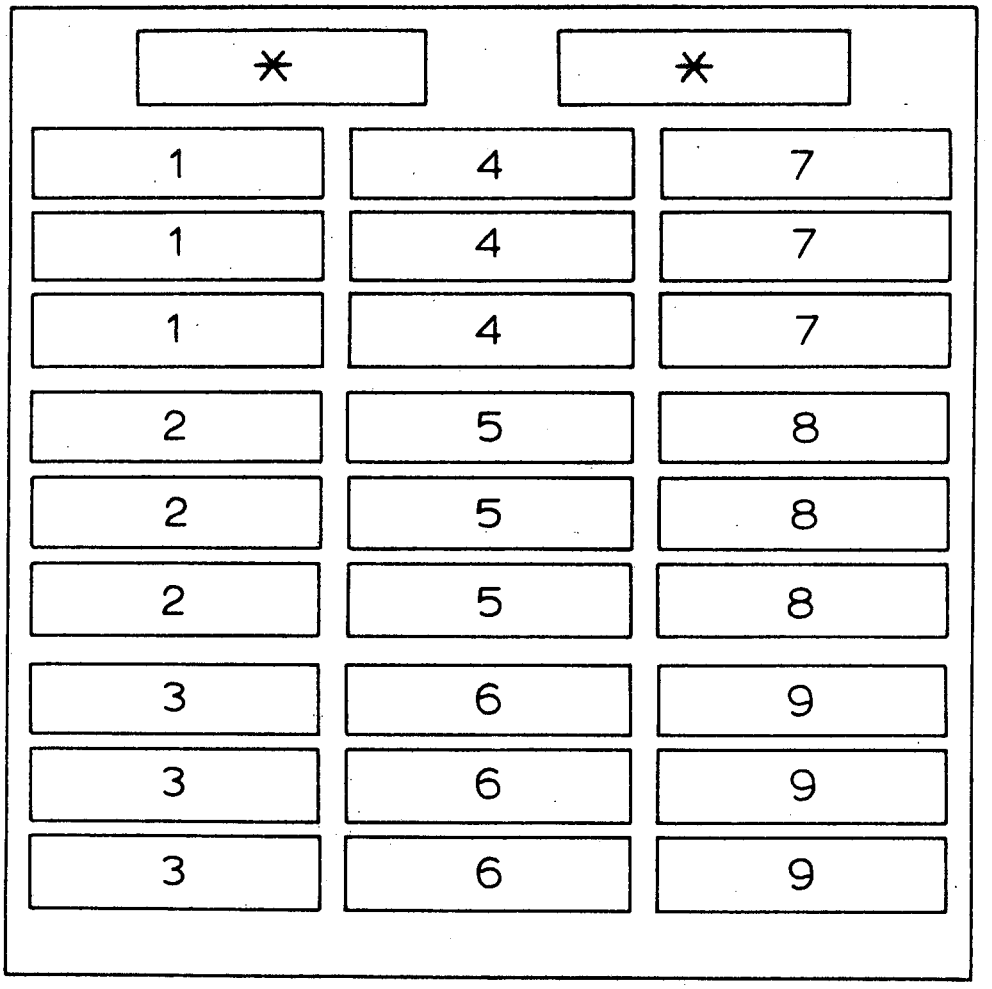

Figure 4 - A sample arrangement of nine groups of boats on the hot plate

6. At removal time the boats were transferred from the hot plate to the center of the cold plate in the same order as they had been placed on the hot plate.
7. After 4 minutes of cooling, the boats were removed and the planchets processed.

Ronald Jacobson

APPENDIX B: SPORE CODES USED AT THE UNIVERSITY OF MINNESOTA

Introduction

The spore code which we use was first discussed in Progress Report 3, December, 1969, pages 76-81. The spore codes developed for organisms studied in the soil experiments are presented below.

Explanation of the Spore Code

The original code consists of four letters. The first letter designates the genus, species and subspecies of the organism if they are known. If the spore is an unknown, any identifying number and the place of isolation are given.

The second letter designates the place from which this laboratory originally obtained the spores.

The third letter identifies the specific spore preparation. Also included under this code letter are the date crop was obtained, cleaning procedures, suspending fluid and any other pertinent information.

The fourth letter designates the storage conditions in this laboratory.

A fifth letter is optional and is used to designate additional information for the unique four letter spore code. A particular fifth letter does not necessarily mean the same thing for different four letter spore codes.

Recently Developed Spore Codes

WACIB, WACIC, WACID

W - Unknown, Cape Kennedy soil

A - Dr. Favero, U.S.P.H.S., Phoenix, Arizona

C - Dry soil received from Phoenix, 12-70 in screw-cap test tube (see Phoenix Quarterly Report 31, July-Sept. 1970, p. 2-3) particle size <43μ

I - 95% ethanol, 4°C

B - Stopped by 10μ filter (>10μ)

C - Passed 10μ filter, stopped by 3μ filter (<10>3μ)

D - Passed 3μ filter (<3μ)

WADI

W - Unknown, Cape Kennedy soil

A - Dr. Favero, U.S.P.H.S., Phoenix

D - Soil in 95% ethanol received from Phoenix, 11-70, stored in freezer until 6-71, particle size <43μ

I - 95% ethanol, 4°C (after 6-71)

WAE1B, WAE1C, WAE1D

W - Unknown, Cape Kennedy soil

A - Dr. Favero, U.S.P.H.S., Phoenix

E - Remaining WAD1 and WAC1 pooled and separated into sizes

1 - 95% ethanol, 4°C

B - Stopped by 10μ filter (>10μ)

C - Passed 10μ filter, stopped by 3μ filter (<10>3μ)

D - Passed 3μ filter (<3μ)

WAH1A, WAH1B, WAH1C, WAH1D, WAH1E

W - Unknown, Cape Kennedy soil

A - Dr. Favero, U.S.P.H.S., Phoenix

H - All available samples that had been separated by sedimentation in size range of 10μ-30μ were pooled

1 - 95% ethanol, 4°C

A - Ethanol removed, resuspended in distilled water, pH 5.5, Bromo Cresol Purple (BCP) added to water, dilute HCl added to retain pH 5.5 after addition of soil. Agitated 72 hours at 4°C. Water removed, resuspended in ethanol.

B - Ethanol removed, resuspended in distilled water, BCP added to water, pH not adjusted after addition of soil. Agitated 72 hours, 4°C, water removed, resuspended in ethanol.

C - Acid added, (similar to A)

D - No acid added (similar to B)

E - No treatment

WA111

W - Unknown, Cape Kennedy soil

A - Dr. Favero, U.S.P.H.S., Phoenix

1 - Received 9-14-71, 100 ml (see Phoenix Quarterly Report 31)

1 - 95% ethanol, 4°C

A - Original sample

XFA1K

X - Unknown, Minneapolis soil

F - Southeast Minneapolis, Dinkytown, collected by Bliss Moore

A - Original obtained 11-17-70, sieved and stored in this laboratory dry until 10-12-71

1 - 95% ethanol, 4°C

K - <43μ

APPENDIX C: SEPARATION OF A SOIL SUSPENSION OF LESS THAN 43 MICRON PARTICLES SIZE INTO <3, 3-10, AND 10-43 MICRON FRACTIONS

To study the dry heat resistance of spores associated with soil particles of a specific size range, we developed the sedimentation procedures described in Appendix D of Progress Report #6. Microscopic examination of the fractions obtained by this procedure, and the experimental results of dry heat resistance studies conducted using these fractions, suggested that the sedimentation technique did not give a precise separation. Therefore we developed a new procedure incorporating both sedimentation and filtration methods. This procedure is shown diagrammatically in Figure 1. The particle size of the fractions obtained was dictated by the pore size of available solvent-resistant filters. Gelman Polypropylene filters (pore size 10 μ) and Gelman Acropor filters (Acrylonitrite Polyvinylchloride Copolymer - pore size 3 μ) were selected. In all of the procedures, several filters of each pore size were used for each filtration to assure that the small sized particles were not trapped in clogged filter pores. The volume of soil-ethanol suspension processed through each filter depended upon the density of that suspension.

10-43 Micron Fraction

One hundred ml of a soil-ethanol suspension were mixed in a 200 ml beaker and allowed to settle for five minutes. The supernatant was decanted and put aside for filtration through the 10 micron filters. After adding ethanol to the sediment in the beaker, the suspension was mixed, settled and decanted. This settling procedure was repeated several times to remove small particles that may have adhered to the larger particles or that may have been carried down with them. The sediment from this primary sedimentation was not included in the 10-43 micron fraction. The decanted supernatant was then put through the 10-micron filters. The filters and particles were insonated in ethanol to remove the particles from the filter surface and to separate adhering particles. The insonated suspension was then placed into 25 x 150 mm screw cap test tubes, shaken and allowed to settle for 30 min. The supernatant was poured off and saved for 10 μ filtration. Ethanol was added to the sediment, the suspension shaken and again settled for 30 min. This 30-minute sedimentation was repeated until the supernatant appeared clear. The supernatant was filtered through the 10-micron filters and the filters were insonated in ethanol to remove the stopped particles. The resulting insonated suspension was combined with the sediment from the 30-minute settling and concentrated by centrifugation.

3-10 Micron Fraction

The filtrate from the 10-micron filtration was filtered through the 3-micron

filters. The soil on the filter surface was removed by insonation in ethanol, and the insonated suspension was placed into 25 x 150 mm screw cap test tubes, shaken and allowed to settle for 18 hours. The supernatant was decanted and saved for 3μ filtration. Ethanol was added to the sediment in the test tube, shaken and settled for 18 hours. This 18-hour sedimentation was repeated until the supernatant appeared clear. The supernatant from the 18-hour sedimentation was filtered through 3μ filters, the soil removed from the filter surface by insonation in ethanol, the insonated suspension combined with the sediment from the 18-hour sedimentation and the resulting suspension concentrated by centrifugation.

3-Micron Fraction

The filtrate from the 3-micron fraction was concentrated by centrifugation.

The fractions obtained by this procedure incorporating sedimentation and filtration methods were examined microscopically and tested for dry heat resistance. Results indicated that the particle size in each fraction was more uniform than in those fractions obtained by sedimentation only.

Geraldine Smith

Q 2.

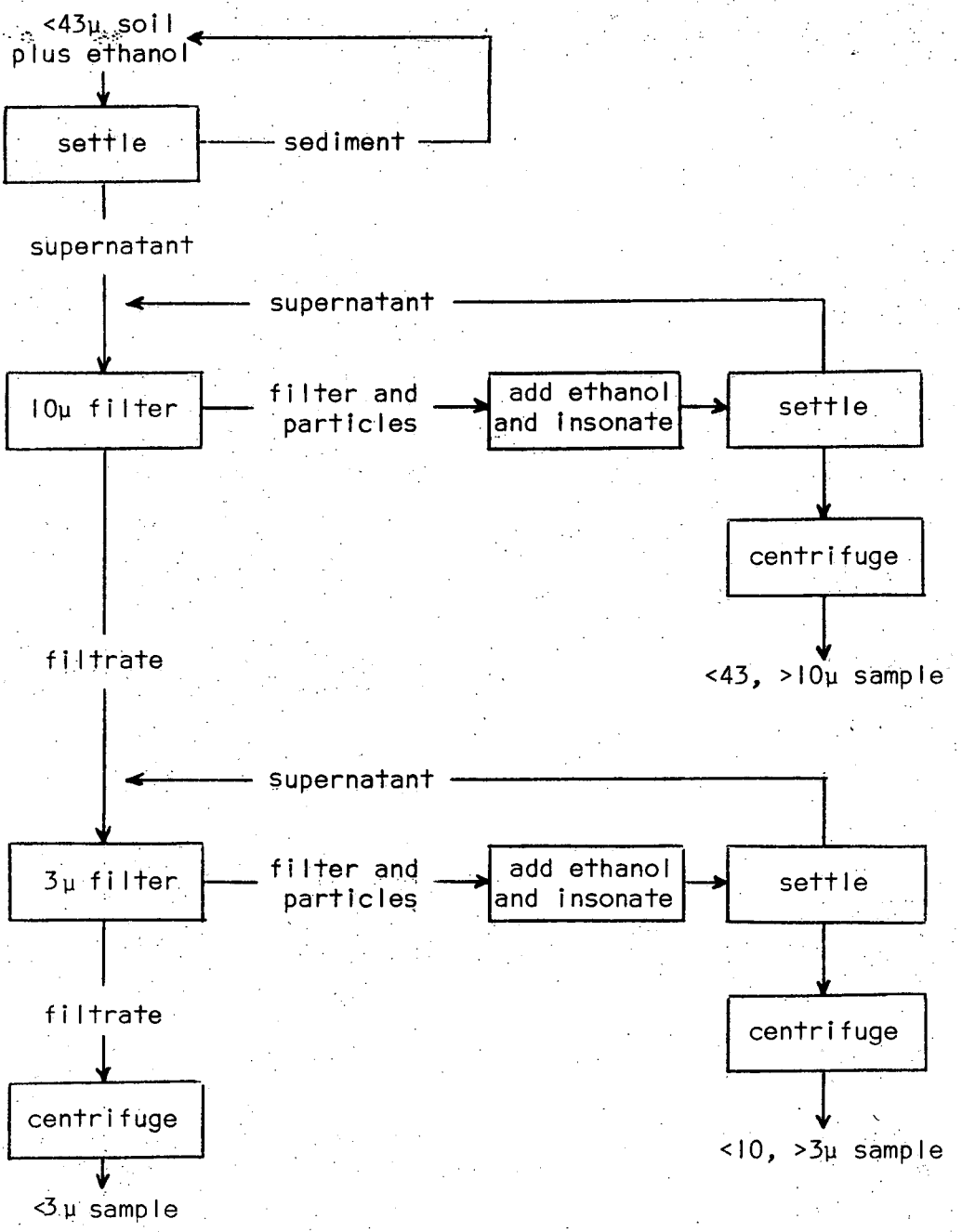

Figure 1 - Diagram of sedimentation and filtration procedures used to separate a soil suspension of <43μ particle size into <3, 3-10, and 10-43μ fractions.

APPENDIX D: EVALUATING THE HEAT BLOCK SYSTEM

Introduction

The heat block system was developed to make it possible to study the destruction characteristics of microbial spores under "closed system" conditions as compared with "open system" conditions. In review, in the open system, water can be lost from the spores and under certain conditions gained by the spores during heating. In the closed system, the water in the system with the spores will be constant, i.e., no gain or loss of water from the time that the system is sealed until the system is opened and the spores are removed.

Some necessary attributes of the units of a closed system are: 1) They should be gas tight; 2) They should heat and cool rapidly; 3) They must be usable for a number of closed system tests; 4) They must be of a design that will permit thorough cleaning; 5) The operations of closing and opening should be rapid and simple; and 6) The cost, in terms of cents per unit test, must be low. It is quite a challenge to design a single system to satisfactorily meet all of these requirements.

In our system we attempted to satisfy the requirements by constructing a stainless steel block unit in which the two halves were bolted together and sealed with a gasket. This gasket has been a source of problems. The first design used an O-ring for effecting the seal between the top and bottom parts of the unit. For a closed system to perform as a closed system with respect to water, there can be no gain or loss of water during heating. When we put our system into operation we found that a Viton O-ring released water during heating. To solve this problem we changed to Teflon O-rings. These O-rings did not perform entirely satisfactorily so we then proceeded to use a flat Teflon gasket. We have now modified our sealing system further by reducing the contact area of the flat Teflon gasket while at the same time increasing the sealing pressure on the gasket. Our microleakage testing program indicates that when using our present sealing system we have a hermetic heat block unit.

In this report we will analyze several different test programs in which the sealing efficiency of the closed block was examined. This activity will be covered in two sections: 1)the testing program and 2) the analysis of microleakage from the heat block system.

1) Testing Program

Two distinct types of tests were used to examine the sealing efficiency of the full-faced Teflon gasket described in Progress Report #5. In one type of test the blocks were loaded with silica gel and heated both in an autoclave and in an

oil bath. Later a group of tests was carried out at initial elevated pressure with the loss of pressure being used as a measure of leakage.

When we found that this full gasket did not perform as well as we expected we then proceeded to modify the gasket and the heat block. Subsequent to this modification, we carried out a retesting program.

Autoclave tests

After thorough cleaning and drying in a 125°C oven for 2 hours, eight stainless steel block units made up of 8 tops (O.D.=2.75", h=.75", central cavity--I.D.=1.185", h=.5") 8 bottoms (O.D.=2.75", central cavity--I.D.=1.28", h=.02") and 48 screws (Allen-head, O.D.=.25, 6/block) were placed in a plastic isolator in which the relative humidity had been adjusted with silica gel to <2% at 22°C. An analytical balance sensitive to 10^{-5} gm, was situated in the plastic isolator, along with dried high-grade silica gel, 8 aluminum boats (L=1", W=.25", h=.25"), 8 small glass vials, a small spatula, forceps and torque wrench.

After an equilibration period of approximately 18 hours, about 0.45 gm of the dry silica gel was placed in each of the 8 boats. Each of the boats was subsequently placed in a particular vial which was then closed and weighed. The boats were removed from the vials; each boat was then placed inside a block unit, which was then bolted shut and torqued to about 50 in-lbs.

Four of the 8 block units were selected at random, removed from the glove box and heated in an autoclave at 125°C (33.7 psia pressure) for four hours. They were cooled slowly for 45 minutes (liquid cool), removed from the autoclave, wiped dry and returned to the plastic isolator. Six hours later, the block units were unbolted and the boats were removed alternately from the control and autoclave blocks and were immediately placed in their original vials which were then closed tightly. Each loaded vial was chosen at random and weighed.

A modified t-test (Behrens-Fisher test) was used for a test of the significance (α=.05) of the difference between means of the two independent samples, treatment and control. Each sample consisted of observed differences in the weight of silica gel before and after conditioning. The estimated probabilities of obtaining larger differences between means of the two samples (assuming no differences) were calculated. In this manner we could ascertain whether a significant amount of water had leaked into the blocks during the autoclaving process.

The changes in the weight of the silica gel in both the control and the autoclave blocks due to the four-hour treatment in the autoclave at 125°C are shown in Table I.

Table 1

Weight Changes(gm) of
Silica Gel in Blocks

Control	Treated
+.00202	+.01095
+.00109	+.01387
+.00133	+.01392
+.00127	+.01424
Mean +.00143	+.01325

The gain in the test system was about 0.012 gm more than the controls. The t-test with α=.05 showed a significant difference between the weight changes of the silica gel in the control and the treated blocks. The conclusion is, that in a four-hour exposure to saturated steam at 125°C (33.7 psia pressure), a small quantity of water does leak into the heat blocks; therefore, they are not truly hermetic.

Oil bath tests

In most of the thermal destruction studies the heat blocks were heated in an oil bath. In this condition the external pressure was about one atmosphere; therefore, the tendency would be for water to leak out of rather than into the blocks, as was the case in the autoclave tests.

An experiment was carried out similar to the autoclave tests except that the atmosphere in the plastic isolator was maintained at a wet condition, 80% relative humidity at room temperature (22°C), in order to load the silica gel with water. The four block units which had been assembled in the isolator were removed from the isolator, heated for 4 hours in the 125°C oil bath and then cleaned and returned to the isolator for weighing. The weight changes for the silica gel in both the control and the heated blocks are shown in Table 2.

Table 2

Weight Changes(gm) of
Silica Gel in Blocks

Control	Treated
+.00363	-.00694
+.00314	-.02326
+.00328	-.03904
+.00216	-.02102
Mean +.00305	-.02257

The mean weight loss of the heated blocks was 0.019 gm, suggesting again that the heat blocks were not hermetic. A t-test (α=.05) showed a significant difference between the weight differences of the silica gel in the control and heated blocks.

Pressure tests

To conduct the microleakage test using the pressure method, a pressure gauge was connected by 1/8" copper tubing to a pressure tap on a standard heating block with a 1/2" recess in the top portion of the block. A special valve and gauge testing unit was used that had a supply port for pressurizing the system with nitrogen gas, a bleeder valve to facilitate the initial pressure adjustment and a gauge to indicate the pressure in the system. A diagram of the apparatus is shown in Figure 1.

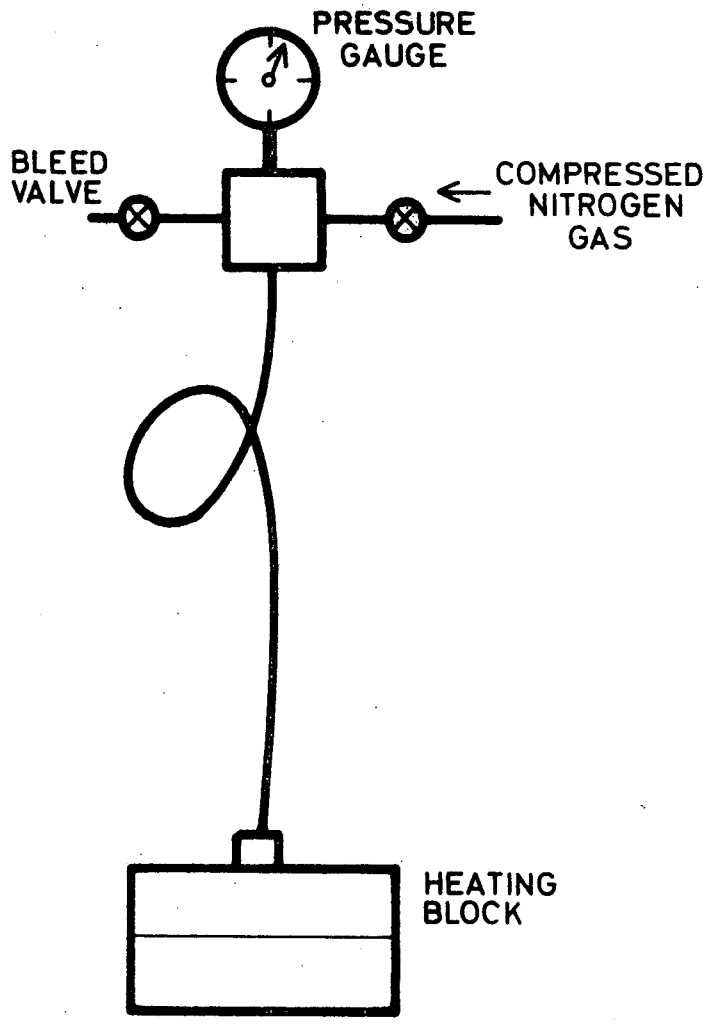

Figure 1 - Schematic diagram of the pressure test system

The clean blocks were assembled with a new Teflon gasket and were torqued to about 50 in-lbs for these tests. The blocks were pressurized to 10 psig at room temperature (22°C) and then were placed into the oil bath at 125°C. (The procedure was similar to that normally employed for thermal resistance testing, and similar to the conditions used in the silica gel-oil bath test described above.) The results of these five different leakage tests are presented in Figures 2 and 3. The blocks were initially pressurized to 10 psig at room temperature and rose to a higher pressure upon immersion in the hot oil bath. The pressure then decayed in the manner shown.

2) Analysis of Microleakage from the Heat Block System

To analyze microleakage from heating blocks two separate situations will be examined and parameterized: 1) leakage from heating blocks during each of the above tests and, 2) leakage from blocks as they are used in the biological testing program.

Analysis of pressure tests

To evaluate the results of the pressure leakage test there are two gas volumes which must be considered: 1) the gas volume in the heating block and that portion of the connecting tube which is submerged in the oil bath and at the oil bath temperature; and 2) the gas volume in the plumbing outside the oil bath which is essentially at room temperature. The respective temperatures and associated volumes are indicated by subscript 1 for the oil bath and subscript 2 for the ambient temperature. Thus the pressure system can be represented schematically by the diagram in Figure 4.

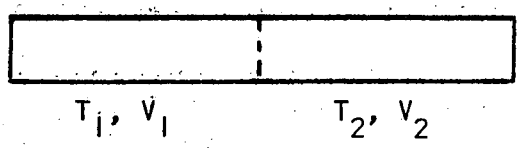

Figure 4 - Modeling diagram of the pressure test system

The system was assembled at temperature T_2 and pressurized to pressure p_o. The block was then placed in the oil bath. Since the gas in volume V_1 was at an elevated temperature T_1, the system's pressure rose to a new higher pressure, p_f. Assuming no leakage during this time and ideal gas behavior of the trapped air, the final pressure will satisfy the following equations:

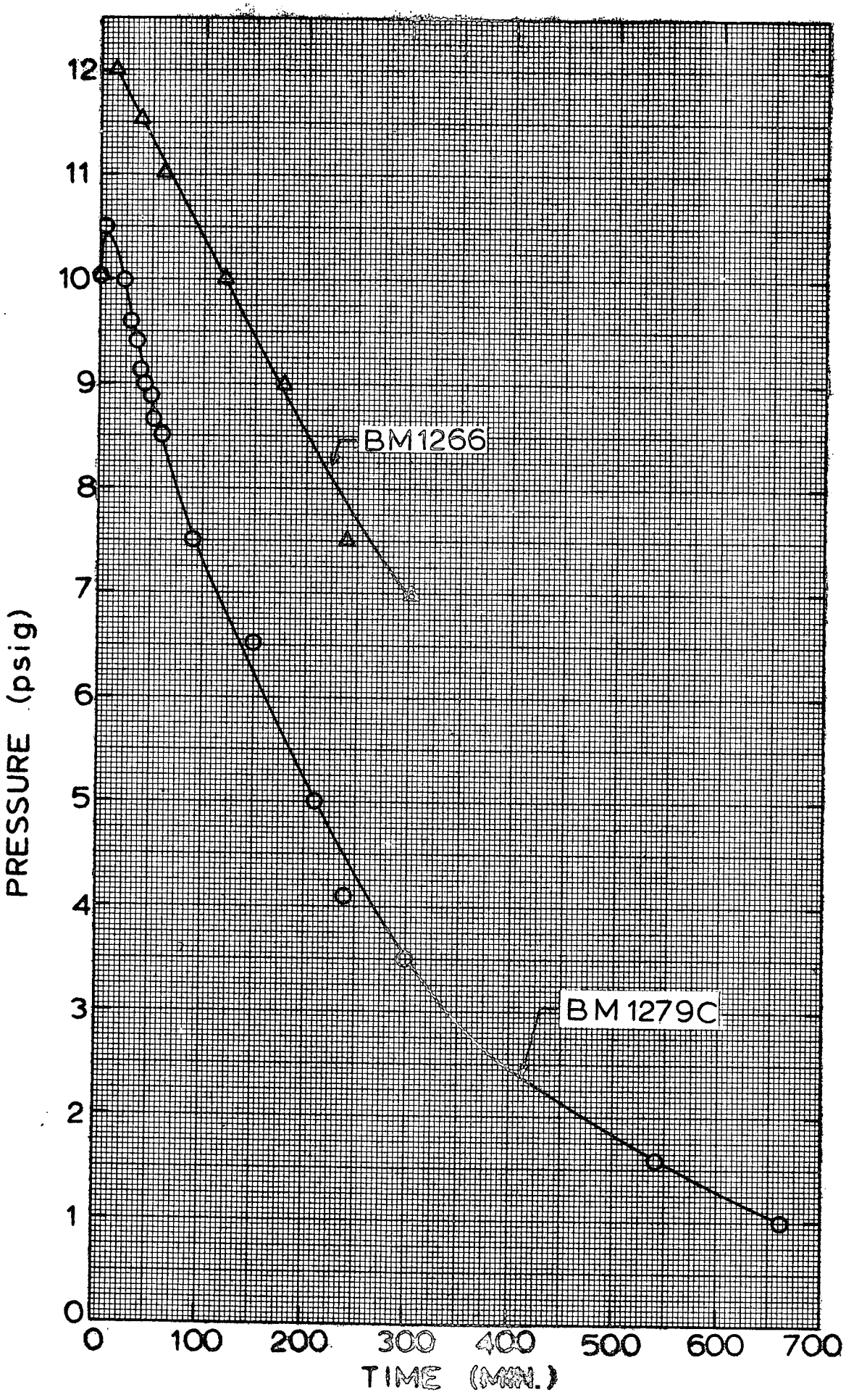

Figure 2 - Results of pressure tests in the oil bath

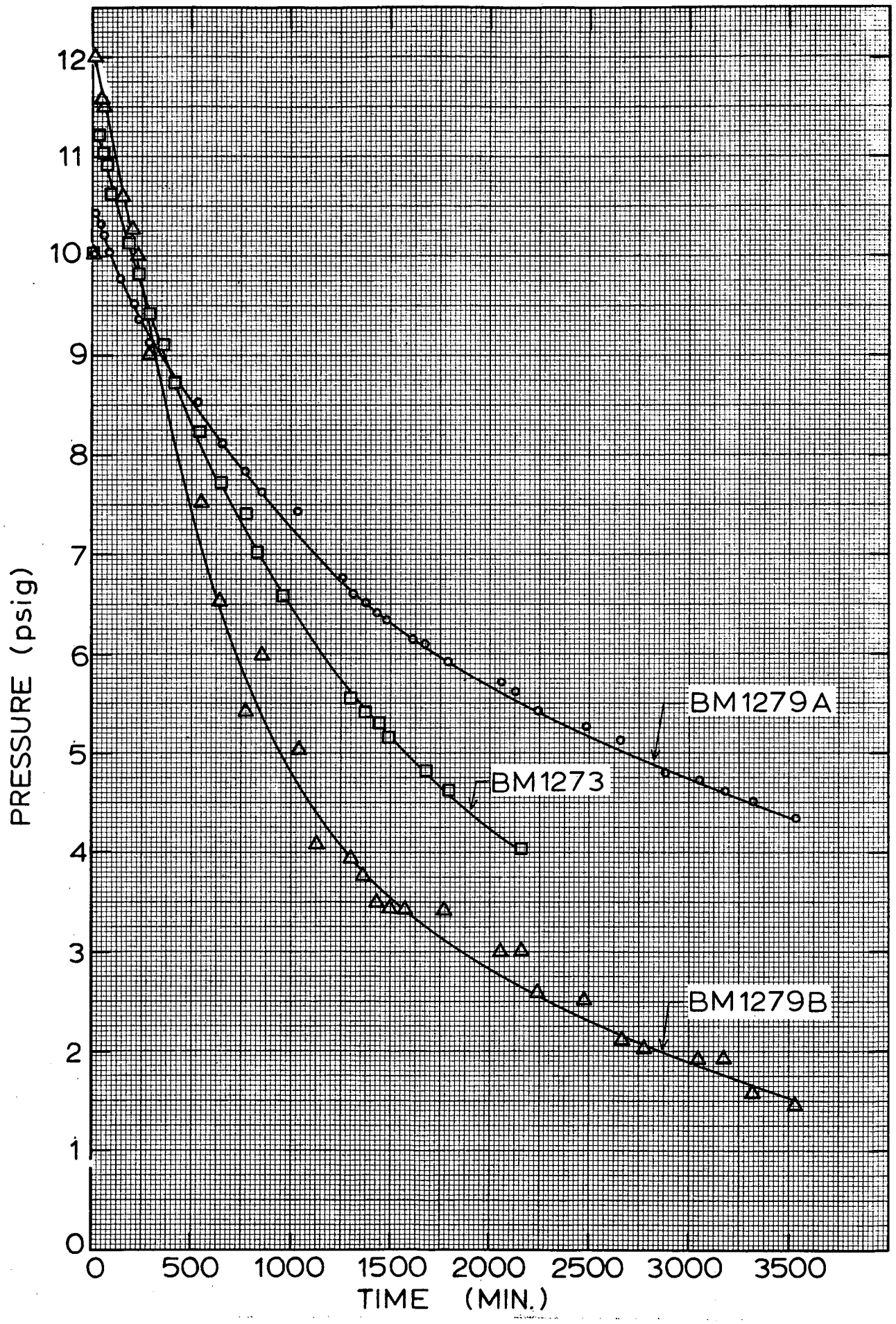

Figure 3 - Results of pressure tests in the oil bath

$$p_o \left[\frac{V_2 + V_1}{T_2}\right] = p_f \left[\frac{V_1}{T_1} + \frac{V_2}{T_2}\right] \tag{1}$$

$$p_f = p_o \left[\frac{(V_1 + V_2)\, T_1}{V_1 T_2 + V_2 T_1}\right] \tag{2}$$

All of the leakage had to have been from volume I through the gasketry. For the assumption of ideal gas behavior (a fairly good assumption for the pressures and temperatures encountered in these experiments) the molar leakage rate will be

$$\frac{dn}{dt} = \left[\frac{V_1}{RT_1} + \frac{V_2}{RT}\right] \frac{dp}{dt} . \tag{3}$$

If we assume that the leakage is large enough so that the flow obeys laws of continuum mechanics, the following analysis can be performed. The flow will no doubt be laminar in view of the low magnitudes of leakage. For isothermal, compressible flow of an ideal gas, the molar flow rate will follow

$$\frac{dn}{dt} = -k(p^2 - p_e^2) \tag{4}$$

where p_e is the pressure at the exit of a leakage pore (essentially atmospheric pressure) and p is the pressure of the inlet, in this case the pressure in the cavity of the closed system. The constant, k, contains such parameters as viscosity, cross-sectional area available for leakage, and geometry, all of which are constant over the duration of one leakage test.

Combining equations (3) and (4), we obtain the ordinary differential equation

$$\left[\frac{V_1}{RT_1} + \frac{V_2}{RT_2}\right] \frac{dp}{dt} = -k[p(t)^2 - p_e^2] . \tag{5}$$

Using the initial condition that $p(t=0) = p_f$ (from equation 2), equation (5) can be solved to give

$$\log \left[\frac{p(t)-p_e}{p_f - p_e} \cdot \frac{p_f + p_e}{p(t)+p_e}\right] = -\frac{2p_e K}{\frac{V_1+V_2}{T_1\ T_2}}\, t \tag{6}$$

where $K = \dfrac{2Rkp_e}{2.303}$.

If we substitute the pressure data from the leakage test into the left-hand

side of equation (6), the results should be linear in time if this model is credible. Figures 5 and 6 show the results from the five leakage tests; the lines on the graph appear to be nearly linear, giving a degree of confidence to the leakage model. The slight curvature of the plot of the results is probably a reflection of entrance effects not accounted for in the model.

Using values of the physical parameters V_1, V_2, T_1, and T_2, the calculated values of K for each of the tests are shown in Table 3. The variation from one test to another is substantial and probably a consequence of change in leakage cross-section between tests.

Table 3

Leakage Constants for Pressure Tests

Test No.	$K \frac{cm^3}{min.^\circ K}$
BMI266	6.014×10^{-5}
BMI273	2.556×10^{-5}
BMI279A	0.849×10^{-5}
BMI279B	1.917×10^{-5}
BMI279C	13.39×10^{-5}
Average	4.945×10^{-5}

Modeling the autoclave leakage test

For the experiment in which the test blocks were heated in an autoclave for four hours we can model the leakage of water vapor into the block cavity from an atmosphere of saturated steam at 125°C. The external pressure is 33.7 psi, the vapor pressure of water at 125°C. Due to leakage, the cavity pressure will asymptomatically approach 33.7 psi.

As before, the molar leakage rate is

$$\frac{dn}{dt} = -k\left[p^2(t) - p_e^2\right] \qquad (7)$$

where k is of the same form as in the pressure tests. Substituting the ideal gas law into equation (7) and integrating, the cavity pressure at time t is

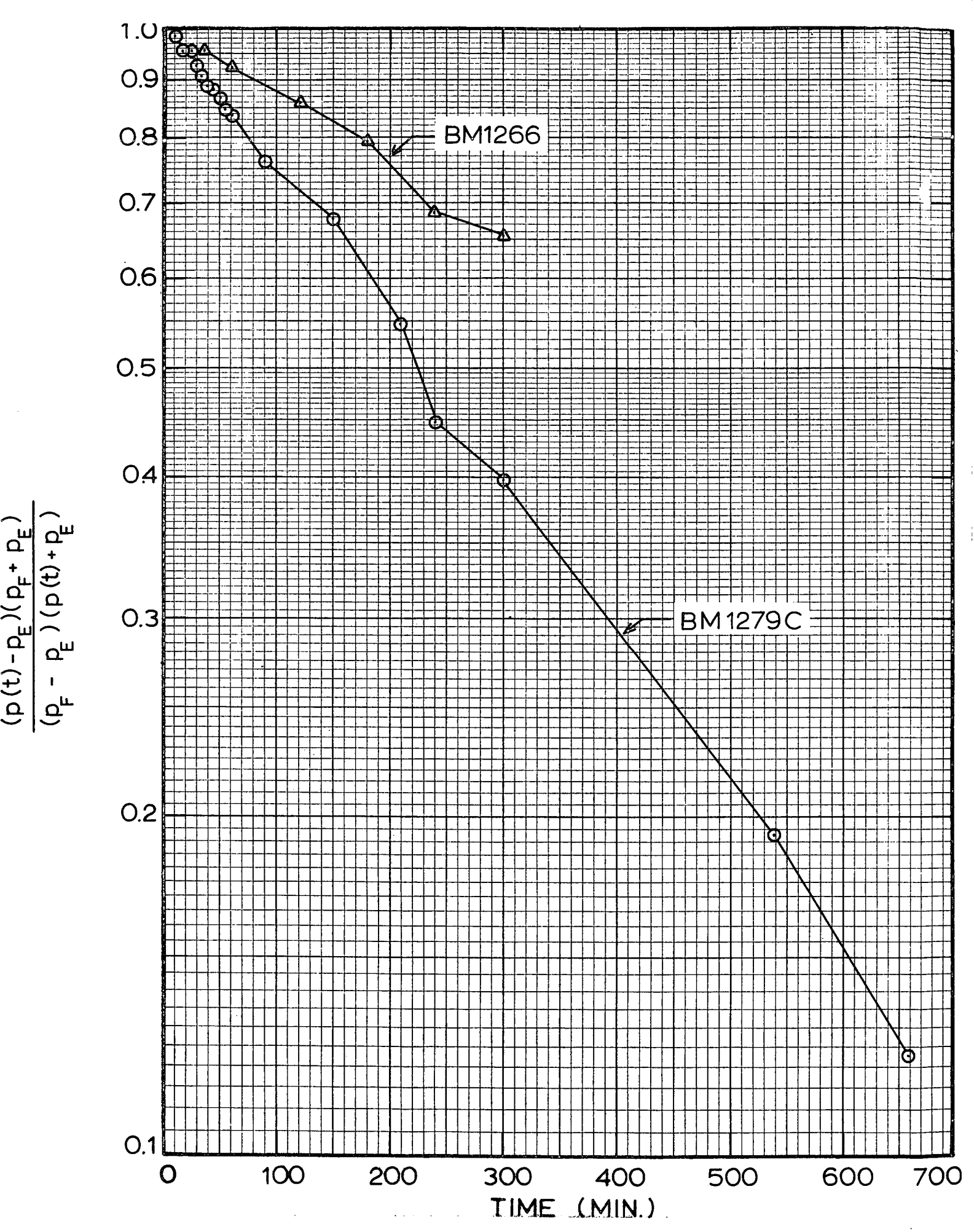

Figure 5 - Plot of transformed pressure data in the form of equation (6)

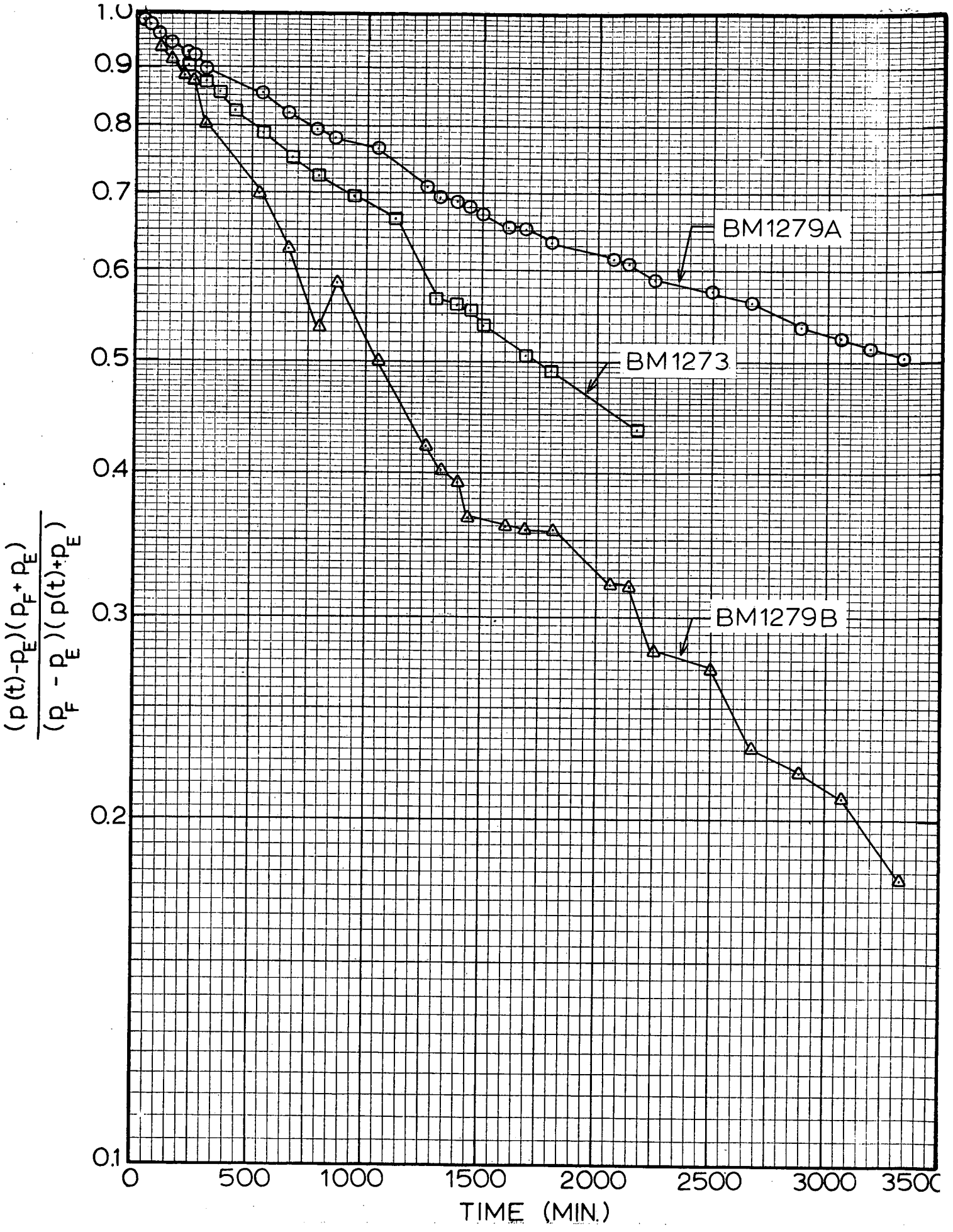

Figure 6 - Plot of transformed pressure data in the form of equation (6)

$$p(t) = p_e \left[\frac{\left(\frac{p_e + p(0)}{p_e - p(0)} \right) \exp\left(\frac{2p_e \, kRT \, t}{V} \right) - 1}{\left(\frac{p_e + p(0)}{p_e - p(0)} \right) \exp\left(\frac{2p_e \, kRT \, t}{V} \right) + 1} \right]. \qquad (8)$$

Substituting for the physical parameters, we find that p(t=24) = 32.53 psi. Thus, the partial pressure of water in the block at this time is 12.70 psi, and the mass of the water vapor is 4.57×10^{-3} gm. After cooling, the water vapor is absorbed into the silica gel and is apparent in the subsequent weighing of the silica gel. The experimentally determined net increase in silica gel weight between treatment and control conditions was .0118 gm. Thus, theoretical prediction and experimental results are within a factor of two of each other which is relatively good when one considers the undefined leakage parameterization and the susceptibility to error of the experimental procedure.

Modeling of the oil bath leakage test

Analysis of this test is possible although a bit more involved than for the autoclave leakage test. In this experiment .45 gm of silica gel is brought to equilibrium with an atmosphere of 80% RH at 22°C. The silica gel is then enclosed in the cavity of a heating block according to the standard sealing technique, and heated in an oil bath to 125°C for four hours.

The microleakage for this test is best analyzed by first focusing our attention on the trapped water. The silica gel contains about 35% or .158 gm water at 22°C. Upon heating to 125°C, most of the water is released by the silica gel and either evaporated or left in the liquid state on the surface of the gel. At 125°C, .0127 gm of water is enough to provide saturated conditions in the block's cavity. Thus there is an ample supply of liquid water to maintain saturated conditions in the cavity even with a fair amount of leakage. The partial pressure of the dry air decreases in time as it is lost from the cavity through microleakage. Thus, the partial pressures in the block are

$$p_{H_2O} = p_{sat}$$

$$p_{air} = z(t) = \text{function of time}$$

To determine the amount of water leakage, we must first determine the rate of loss of dry air and thus the decrease in total pressure in the cavity. After we

know the total pressure we can determine the overall leakage rate and, thus, the water vapor leakage. As before, the molar leakage rate is

$$\frac{dn}{dt} = -k\left[p_{total}^2 - p_e^2\right]$$

$$= -k\left[(p_{sat} + z(t))^2 - p_e^2\right] \tag{9}$$

where k is the same leakage constant that was used in the pressure experiments. The molar rate of leakage of dry air is

$$\frac{dn_{air}}{dt} = \frac{p_{air}}{p_{total}} \frac{dn}{dt} \tag{10}$$

Combining equations (9) and (10) and substituting the ideal gas law we find

$$\frac{dz(t)}{dt} = -\frac{kRT}{V}\left[\frac{(p_{sat} + z(t)^2) - p_e^2}{p_{sat} + z(t)}\right] z(t) \tag{11}$$

which after integration and use of the initial value of z(0), shows

$$\frac{kRT}{V} t = \frac{1}{2(p_{sat} + p_e)} \ln\left[\frac{z(0) + p_{sat} + p_e}{z(t) + p_{sat} + p_e}\right]$$

$$+ \frac{1}{2(p_{sat} + p_e)} \ln\left[\frac{z(0) + p_{sat} - p_e}{z(t) + p_{sat} - p_e}\right]$$

$$+ \frac{p_{sat}}{2(p_{sat}^2 - p_e^2)} \ln\left[\frac{z(t)}{z(0)}\right]. \tag{12}$$

With this implicit solution for the partial pressure of the dry air in the blocks, we now have a formulation for the total pressure as a function of time and, consequently, can determine the overall molar leakage rate. Thus, the molar leakage

rate of water is

$$\frac{dn_{H_2O}}{dt} = - \frac{k\, p_{sat}}{p_{sat} + z(t)} \left[(p_{sat} + z(t))^2 - p_e{}^2\right] \qquad (13)$$

and the mass of water lost over the 4-hour treatment period is

$$m_{H_2O} = \int_0^{240} 18\, k \frac{p_{sat}}{p_{sat} + z(t)} \left\{\left[p_{sat} + z(t)\right]^2 - p_e{}^2\right\} dt . \qquad (14)$$

Since we have only an implicit solution for z(t), this integral can be best calculated numerically. Such a calculation predicts that .018 gm of water should be lost during the treatment period. The experimentally determined net decrease of treated over control samples shows an average loss of .022 gm.

Since water vapor is only .61 as viscous as dry air at 125°C, the parameter k should be weighted to show its dependency on viscosity, and, therefore, gas composition. As a first approximation, we can say that the viscosity of the gas mixture is equal to the mole fractional contributions of the component gases. Thus, the leakage factor should be for the form

$$k_{actual}(t) = \frac{k}{\dfrac{z(t) + .61 p_{sat}}{z(t) + p_{sat}}} . \qquad (15)$$

Solving a corrected form of equations (12) and (14), the predicted amount of water loss is .0247 gm.

We believe that this is in rather good agreement with the experimentally measured loss of .022 gm.

Biological experimental conditions

When the blocks are being used to test bacterial spore survival, the captive air volume is smaller since no connections to the pressure gauges have to be made and all of the gas is at temperature T_1. In this analysis, let the primed parameters denote physical properties during the biological experiments.

The blocks are assembled at temperature T_2, and then immersed into the constant temperature bath at T_1. Thus, the pressure rises following the ideal gas law to a higher pressure as a consequence of the temperature rise. Let the time

origin be the time when the block reaches temperature T_1. Thus, the pressure at zero time will be

$$p'(t=0) = (1\ ATM)\frac{T_1}{T_2}. \tag{16}$$

As was the case in the pressure tests the rate of pressure decay is related to the molar flow.

$$\frac{dp'}{dt} = \frac{RT_1}{V'}\frac{dn'}{dt} \tag{17}$$

Since the same full-faced gasketry and the same bolt torque were used, the same leakage geometry and flow conditions should exist. The leakage rate can be described as

$$\frac{dn'}{dp} = k\,(p'(t)^2 - p_e^2) \tag{18}$$

where the constant k is the same as in equation (5). Combining equations (17) and (18) and solving the subsequent differential equation, we get the expression

$$\log\left[\frac{p'(t) - p_e}{p'(0) - p_e}\cdot\frac{p'(0) + p_e}{p'(t) + p_e}\right] = \frac{-k\,t}{\frac{V'}{T_1}}. \tag{19}$$

Using the average value of k from Table 3 and physical measurements from the various block systems, the absolute pressure during the biological tests would satisfy

$$\frac{p'(t) - p_e}{p'(0) - p_e}\cdot\frac{p_f + p_e}{p(t) + p_e} = \begin{cases}\exp(-.05549t) & \text{for flat top blocks} \quad (20a)\\ \exp(-.01536t) & \text{for 1/8" recess blocks} \quad (20b)\\ \exp(-.00870t) & \text{for 1/4" recess blocks} \quad (20c)\\ \exp(-.00458t) & \text{for 1/2" recess blocks} \quad (20d)\end{cases}$$

We can rearrange equation (11) to give an expression for the absolute pressuee,

$$\frac{p'(t)}{p'(0)} = \frac{1 + \frac{p_e}{p'(0)}\,\frac{1-E}{1+E}}{1 + \frac{p'(0)}{p_e}\,\frac{1-E}{1+E}} \tag{21}$$

where $E = \exp\left[\frac{-2.303\ k\ T\ t}{V'}\right]$.

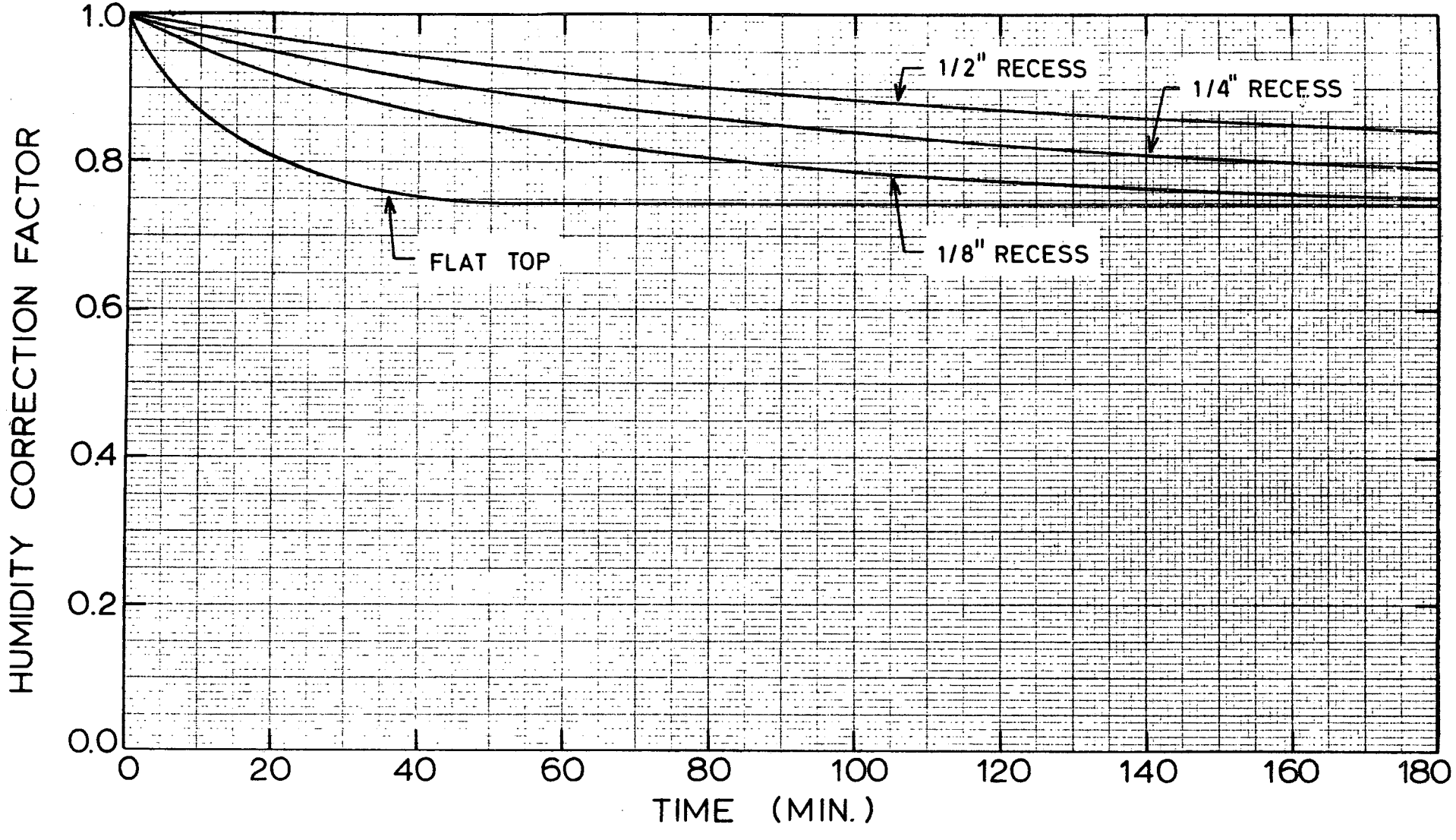

Figure 7 - Humidity correction factor for various block recesses using the full-faced gaskets during biological tests

Since the leakage satisfies a model based on a continuum scale of gas flow, each component gas will escape in proportion to its bulk concentration. The mole fraction of the water vapor will remain constant in the gas space of the block. The partial pressure, p_{H_2O}, and the relative humidity will be functions of time. This becomes apparent if we manipulate the left-hand side of equation (21).

$$\frac{p'(t)}{p'(0)} = \frac{p'(t)\ y_{H_2O}}{p'(0)\ y_{H_2O}} = \frac{p_{H_2O}(t)}{p_{H_2O}(0)} = \frac{p_{H_2O}(t)/p_{sat}}{p_{H_2O}(0)/p_{sat}} = \frac{RH(t)}{RH(0)} = \frac{RH(t)}{RH_{reported}} . \quad (22)$$

Figure 7 shows how this correction factor for relative humidity changes for each block configuration during the course of an experiment. For the flat-top heating block, the relative humidities very quickly approached a value of 75% the initial relative humidity. For the 1/8", 1/4", and 1/2" recessed blocks, the approach to the same asymptote is progressively slower.

Modified flat Teflon gasket with increased torque of cap screws

The sealing system of the heat block was modified to reduce microleakage; the area of the flat Teflon gasket was reduced from 4.79 to 0.79 in^2 and the cap screw torque was increased from 50 to 100-120 in-lbs. These modifications increased the gasket sealing pressure by a factor of 12. A drawing of the modified block is shown in Figure 8. The new gasket has dimensions of O.D.=1.625", I.D.=1.28", and d=.031". The ring is fitted into a slight recess in the top portion of the block which holds the gasket during the assembly of the block. This method of holding the gasket facilitates alignment as the blocks are being assembled.

The modified gasket system was pressure tested to determine the effect of the alterations on the sealing efficiency. We felt that the pressure test offered the most direct method for detecting microleakage. The blocks were pressurized in the manner previously used for testing blocks with standard gasketry except that the initial pressure was 50 psig instead of 10 psig. To date six tests have been carried out. In five cases there was no detectable drop in pressure over the 24-hour period that the blocks were in the oil bath at 125°C. One block showed a drop of 4 psi in 24 hours. It is possible that this leakage occurred in the tubing connections rather than the heat block gaskets. The testing program is continuing.

Summary

Gas leakage from pressurized heating blocks has been modeled and experimental data seems to indicate that the model is reasonable. An appreciable amount of

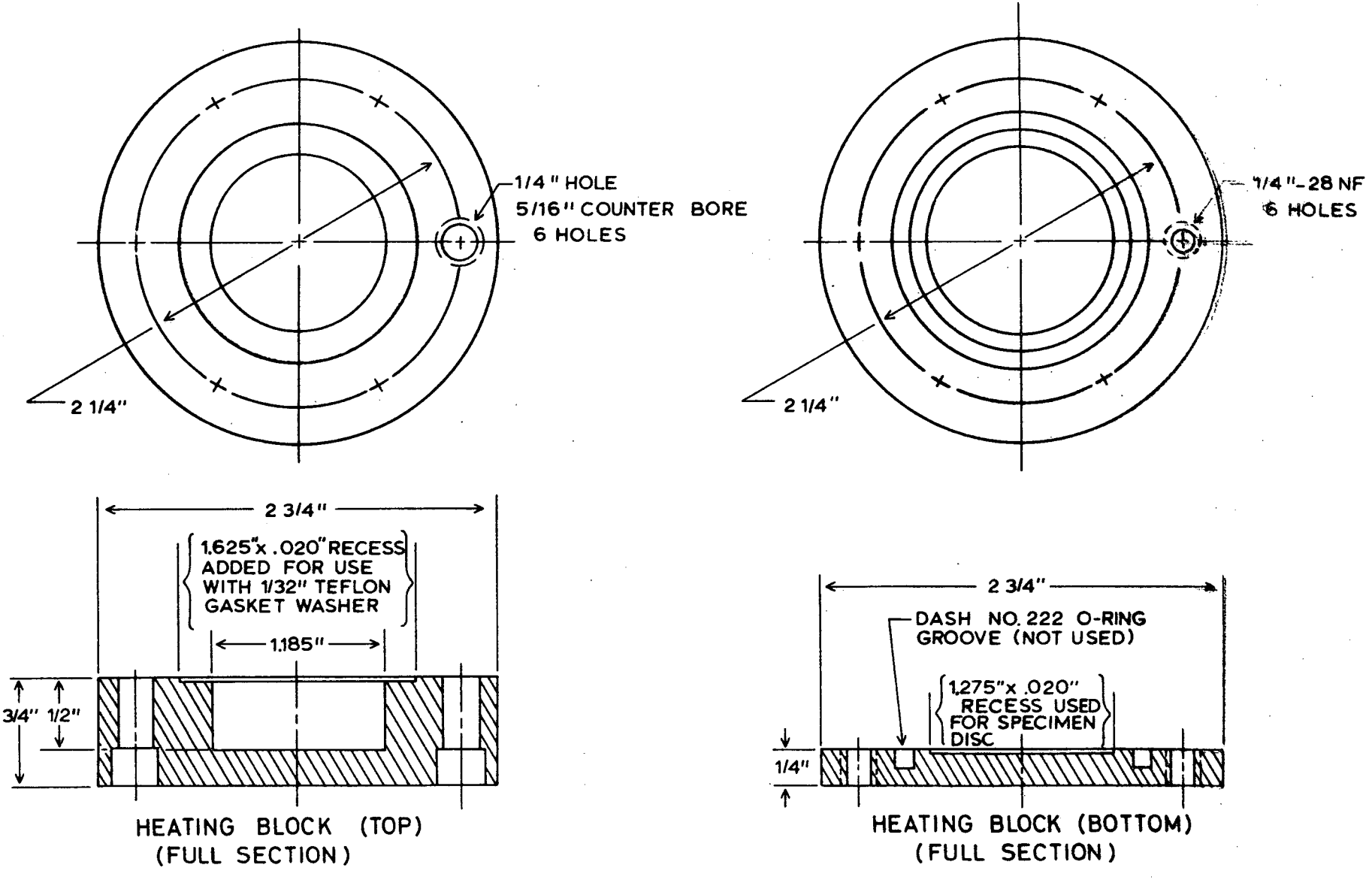

Figure 8 - Drawing of the modified closed block components

deviation from one run to another is evident. Cross correlation between different leakage tests is fairly consistent. Time profiles for a representative leakage rate are shown for such environmental properties as pressure, water vapor pressure, and relative humidity.

A new gasket system has been developed and is being used where microleakage is below the level of measurement when a pressure of 50 psi acts for 24 hours with the block in the 125°C oil bath. Since this pressure is in general at least two times the maximum pressure in the heat block during testing and the heating times at this pressure usually do not exceed 8 hours, we believe that we can proceed to use the heat block system with confidence that there is no significant leakage. A regular test program will be instilled to insure that the leakage rates remain at these low levels.

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